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to

The National Toxicology Program

- Appendix 6 -

Measurement of Cotinine Concentration in Biological Fluids and Use of the Results in Epidemiological and Exposure Studies

March 20, 1998

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Introduction

There are two reasons that a biomarker for tobacco use or environmental tobacco smoke (ETS) exposure is of considerable interest. The first is to determine whether an individual is a smoker or nonsmoker. The second is to attempt to quantify levels of exposure to ETS for nonsmokers. To answer the first question is relatively straightforward, since in principle the difference between a given biomarker for a smoker and a nonsmoker should be large. The second question, however, requires one to attempt to differentiate between two small and similar values. As a result, the analytical criteria for these two applications are very different, and require consideration of different methodologies. In addition to the analytical concerns, the potential impact of interpersonal variation in metabolic rates and pathways contributes confusion. Add the choice of saliva, serum, or urine as the sample matrix, and it is easy to see why a complex set of measurement and data interpretation uncertainties has evolved. Some of these issues will be addressed in this discussion.

Cotinine is a carbon-oxidation metabolite of nicotine. It is found at measurable levels in saliva, serum and urine of subjects who smoke, who use other forms of tobacco, or who have been exposed to ETS at sufficient levels (Benowitz, 1996). Cotinine meets many of the criteria proposed by the National Research Council (NRC) for an ETS biomarker (NRC, 1986), but it must be kept in mind that cotinine is derived from nicotine. Although cotinine may serve as a biomarker for nicotine intake, it may not serve as well as a biomarker for ETS exposure. Furthermore, cotinine has a biological half-life of approximately 20 hours and can only represent nicotine intake within an

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interval of a few half-lives. While a number of alternative biomarkers have been proposed, most of these have been devaluated relative to cotinine (Benowitz, 1996, Table 5). In the search for a biomarker for tobacco use or ETS exposure, by a process of elimination cotinine appears well on the way to becoming the compound of choice for many researchers (Benowitz, 1996).

Analytical methods may be described as selective or as specific. Although many analytical methods exist for specific detection and quantification of cotinine, most of these are based on instrumental methods of analysis (Benowitz, 1996, Table 4). Frequently, these methods are considered too expensive, too labor intensive, or to require special skills beyond those frequently available for epidemiological or exposure studies. Radioimmunoassay (RIA) methods for cotinine were developed and widely applied in the scientific community that conducts various epidemiological and exposure investigations (Langone et al., 1973). RIA methods are rapid, economical and require few special analytical skills, but they are selective rather than specific for chemical compounds.

Selectivity was likely thought to be an adequate criterion for cotinine. Until approximately 1987, cotinine was considered to be the major, if not the only, known nicotine metabolite of analytically significant concentration in the biological fluids of interest. Although N-oxidation metabolites of nicotine were known, they were found only at low concentrations (Turner, 1969). After 1987, the presence of significant concentrations of *trans*-3-hydroxycotinine (Neurath and Pein, 1987), and glucuronide conjugates of nicotine, cotinine and *trans*-3-hydroxycotinine (Curvall et al., 1989) in urine were recognized. Although cotinine appears to be the major nicotine

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metabolite in saliva and serum (Curvall et al., 1990), *trans*-3-hydroxycotinine (Byrd et al., 1994) or its glucuronide conjugate (Andersson et al., 1997) is the most prevalent in urine. Actually, cotinine constitutes only 10-15% of the nicotine metabolites. At least seventeen (17) metabolites of nicotine have been identified in urine (Kyerematen et al., 1987). Many of these have been quantified (Byrd et al., 1994; Andersson et al., 1997).

Cotinine as a Biomarker for Smoker Classification

The evolution of cotinine as a biomarker began as a means to discern smokers from nonsmokers. The levels of cotinine in saliva, serum and urine of a smoker of >20 cigarettes per day is relatively easy to distinguish from that of a nonsmoker using a variety of analytical methods including RIA. In large epidemiological studies for which cost, labor, time and other issues are important, saliva samples are the easiest to obtain. It has been reported that analytical determinations of cotinine in saliva provide essentially the same information as similar determinations in serum (Curvall et al., 1990). A mean saliva to serum ratio of cotinine concentration of 1.2 has been reported (Curvall et al., 1990). Although further study is needed, at this time both matrices are thought to be relatively free of other analytically significant metabolites of nicotine. The principal reason that nicotine is not used as a biomarker for its uptake is that the half-life of nicotine in biological fluids is only approximately 2 hours (Benowitz, 1996). Measurement of nicotine would not give a representative estimate of nicotine intake during the previous few days.

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In epidemiological or exposure studies, classification of an individual as a smoker or nonsmoker is critical to the results, especially in the interpretation of data at low relative risk (RR) levels such as those reported with regard to ETS exposure (Jenkins et al., 1996). Wide ranges of smoking status misclassification have been suggested based on questionnaire data (Riboli et al., 1995), and misclassification of 2 -5% smokers claiming to be nonsmokers is not uncommon (Jenkins et al., 1996; Phillips et al., 1996). RR values below approximately 2.0 are considered weak associations (Greenberg, 1986). In the area of ETS epidemiology, low RR values, as for example 1.2, could be significantly influenced by smoking status misclassification of less than 5%.

Utilization of cotinine as a biomarker for smoker classification is less of an analytical issue than a policy issue. Interpersonal variability in metabolic processes, lifestyle, and other factors creates variations in cotinine levels that make it very difficult to distinguish an occasional smoker from a nonsmoker with recent exposure to high levels of ETS (Phillips et al., 1996). An additional issue that is rarely addressed in the literature is "intra-personal" variability. For example, researchers who conduct exposure studies infrequently collect more than one, possibly two, samples from an individual subject. Ranges of exposure that result from daily lifestyle variations are not considered. This variability may well be much greater than the analytical variability.

As a result, uncertainty exists over the cotinine concentration that unambiguously differentiates a smoker from a nonsmoker. At the present time, somewhat empirical decisions are made. These decisions may be influenced by the goal of the investigation. It has been suggested that cotinine concentrations of 10-50 ng/mL in saliva or serum, or 50 nanogram cotinine per milligram

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creatinine in urine (cotinine-creatinine ratio: CCR) are levels below which a subject is considered to be a nonsmoker and above which is classified as a smoker. Conflicts with questionnaire data arise and are occasionally resolved by dismissing the subject from the study. The latter decision is prudent for experimental purity, but could eliminate an important segment of the total population.

The complex distribution of cotinine species in urine would recommend the use of serum or saliva for smoking status classification. Pirkle et al. (1996) have determined serum cotinine levels for over 4,000 subjects spanning wide age groups, smoking status and ETS exposure. Their results show a finite percentage of the population with serum cotinine levels below 10 ng/mL that report tobacco use, and above 10 ng/mL that report no tobacco use or ETS exposure at home or at work. Three regions of classification should be considered: smokers, nonsmokers and indeterminate. The overlap is a combined result of interpersonal metabolic variances, variation in the degree of ETS exposure in nonsmokers, inaccurate questionnaire-derived data and analytical variance. These levels of uncertainty not only have potential for impact on conclusions drawn from scientific studies, but on decisions regarding individuals such as classification for other purposes.

In summary, when an individual is clearly a smoker, or clearly a nonsmoker with moderate ETS exposure, cotinine measurements in saliva, serum or urine may be capable or distinguishing between the two. To determine the classification of occasional smokers or nonsmokers with significant ETS exposure is not an analytical issue, but one of establishing a decision point. Establishing this point is complicated by the lack of a clear definition of a smoker. If the cotinine level used to classify a subject as a smoker is too high, smokers may be erroneously

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classified as nonsmokers. One study (Saracci and Riboli, 1989) that classified as smokers subjects with urinary levels of cotinine above 50 ng/mg creatinine, later reported that of the 47 subjects that were excluded from the study as smokers, only 20 (1.5% of the total study population) can be considered to be smokers (Riboli et al., 1995). The authors suggested that a cutoff level of 150 ng/mg should be used to avoid exclusion of nonsmokers with high ETS exposure. The position could be taken that a level lower than 50 ng/mg is more realistic to identify light smokers who are deceivers in questionnaire responses. This example clearly demonstrates that a single point value used for all subjects is woefully inadequate for such an important decision. Selection of the point value criteria may be driven inadvertently by the goal of the study.

Cotinine as a Biomarker for ETS Exposure

The analytical chemistry issues for the use of cotinine as a biomarker for classification of smoking status are minor compared to those associated with its use for estimating ETS exposure-dose relationships. The evolution of the use of cotinine levels from a method to make a binary decision (smoker or nonsmoker) to implied quantification of ETS exposure was a slow but relentless process. Cotinine concentrations in the case of regular smokers are normally 2-3 orders of magnitude greater than of the limit of quantification (LOQ) of analytical methods that have been applied. Even the marginal region of 1-5 ng/mL in saliva, serum and urine are adequately measurable using RIA and chromatographic methods.

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Especially in urine samples, analytical and interpersonal metabolic considerations may become paramount. These factors, coupled with what may be considered a propensity toward analytical expediency in epidemiological ETS exposure studies, may lead to opportunities for contradictory conclusions. Recently, prominent professors of epidemiology expressed the view that contradictory findings in epidemiological research are "common" (statements of Trichopoulos et al. 1997). It is possible that some of these contradictions stem from inadequate experimental design, insufficient number of subjects, or from errors in the analytical data upon which decisions are based.

As mentioned above, saliva, serum and urine have all been used as matrices for the determination of nicotine and its metabolites. Because urine has been used in a large number of epidemiological studies, and because that matrix appears to contain the largest number of metabolites, it will be considered first.

Metabolic Products of Nicotine in Urine

At least 17 metabolic products of nicotine have been identified in human urine (Kyerematen et al., 1987). Of these, cotinine has been the focal component. Analytical methodologies have been developed that may be applied reasonably to those compounds shown in **Table 1**, along with some of their glucuronide conjugates. The remaining metabolites require more involved analytical methods. **Table 2** shows the results of two independent studies that measured in human urine the compounds shown in **Table 1**. The results are reported as percent of the total found based on nicotine equivalents of each compound, and the cumulative total if the compounds

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Table 1. Nicotine and metabolites that have been determined analytically.

Compound	Abbreviation
Nicotine	NIC
Nicotine glucuronide conjugate	NIC-G
Cotinine	COT
Cotinine glucuronide conjugate	COT-G
trans-3'-Hydroxycotinine	3HC
trans-3'-hydroxycotinine glucuronide conjugate	3HC-G
Nicotine-N'-oxide	NNO
Cotinine-N-oxide	CNO
Demethynicotine	DMC

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Table 2. **Distribution of nicotine and metabolites in two studies. (Each compound was converted to an equivalent amount of nicotine.)**

NIC + metabolites	% of total found[1]	cumulative % total	% of total found[2]	cumulative % total
3HC	35	35	36	36
COT-G	17	52	14	50
COT	13	65	9	59
NIC	10	75	9	68
3HC-G	9	84	23	91
NIC-G	3	87	5	96
NNO	7	94	3	99
CNO	4	98	1	100
DMC	2	100	Not Determined	
(Smokers) N =	11		91	
PERCENT N-OXIDE	11		4	

1. Byrd et al. (1994) (SD values are 3-8%, taken from Table I, RSD values calculated to be 21-75%).

2. Andersson et al. (1997) (SD values are 0.9-10.6 taken from Table 4, RSD values calculated to be 28-100%).

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are taken into account sequentially. There is general agreement between these mean values reported in the two studies, although a significant difference was found between the distribution of the glucuronide conjugates, especially the 3HC-G. The variance of the mean of the percentage of each metabolite is somewhat higher in the study by Andersson et al. (1997) than in the one by Byrd et al. (1994).

It is clear from **Table 2** that free (i.e., not conjugated) cotinine accounts for only about 10% of the total nicotine metabolites analytically detectable in human urine. This observation requires a number of considerations:

1. Whether a selective (i.e., RIA) or specific (i.e., GC/MS) analytical method is used for cotinine determination, only about 10% of the nicotine equivalent as cotinine will be measured unless an enzyme-catalyzed hydrolysis is conducted to free the conjugated cotinine. Even then only a portion of the nicotine metabolites is measured.

2. In the case of RIA, a cross reactivity of approximately 34% for *trans*-3'-hydroxycotinine (3-HC) in urine samples has been reported (Zuccaro et al., 1997). Multiple regression of data presented by Zuccaro et al. (1997) yields an expression for cotinine concentration determined by RIA ($[COT]_{RIA}$) versus cotinine concentration ([COT]) and trans-3'-hydroxycotinine ([3-HC]) determined by HPLC as follows (concentrations in μg/L):

$$[COT]_{RIA} = 0.97\pm(0.63)\,[COT] + 0.29(\pm0.14)[3\text{-}HC] + 557(\pm371)$$

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Interestingly, both the coefficient of variation and the "p" value for the coefficient for 3-HC are smaller than that for COT. This suggests that the results from RIA determinations as used by Zuccaro et al. are more strongly correlated to the [3-HC] than to [COT], although more selective for cotinine.

The results of both studies represented in **Table 2** indicate that the mean *trans*-3'-hydroxycotinine is about three times the concentration of cotinine in urine. RIA would yield a result that is a combination of cotinine and cross reactivity with *trans*-3'-hydroxycotinine. The analytical bias can be as high as a factor of two, and leads to considerable confusion when RIA results are compared to GC or GC/MS results.

3. If enzymatic hydrolysis of the conjugates is included in the analytical step, the resulting nicotine, cotinine and *trans*-3'-hydroxycotinine concentrations determined by chromatography could account for approximately 85-90% of the total nicotine and its metabolites found in urine samples. This can be concluded from the cumulative total columns in **Table 2**. The only reliable approach to relate nicotine dose (and, thereby, uptake) to nicotine metabolites in urine for individuals is to perform a complete suite of metabolite determinations to account for pharmacokinetic differences.

4. The results in **Table 2** represent mean values that are reasonably consistent between the two studies. However, the variation of each mean can be up to 100%. Some of this distribution is analytical error; some of it is interpersonal variation. Although correlation between means

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in different matrices has been established (Benowitz, 1996), no controlled experiments are known to determine the variance between individual values either on an interpersonal or intrapersonal level.

In summary, with frequently used analytical approaches for determination of cotinine in urine, only about 10% of the total nicotine is accounted for in the analysis. When RIA is used, the representation as a cotinine determination may be in error by as much as a factor of two. Approximately 25-35% of the metabolites is present as conjugates not detected unless enzymatic hydrolysis is conducted. Interpersonal variation is an important issue dealing not only with nicotine exposure, but also with metabolic rates and distribution among the various metabolites and their conjugates. When only 10% of the metabolite products of nicotine are used, it is not unexpected that large variations in results will be obtained.

Finally, urine volume is a variable associated with many factors including liquid consumption. It is possible that careful management of 24-hour urine sampling can be used successfully (Benowitz et al., 1997). Unfortunately, many studies related to ETS exposure make determinations using only one sample. Use of the cotinine-creatinine ratio (CCR) to adjust for urine output has limitations because of the variables that influence creatinine output.

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Metabolic Products of Nicotine in Serum and Saliva

Little has been reported in the literature concerning nicotine metabolites other than cotinine in blood. From the point of view of analytical significance, cotinine appears to be the most important nicotine metabolite in serum. The conversion of nicotine to cotinine in blood by the liver of smokers has been related to daily nicotine intake as 0.08 [(mg/24 hr)/ng/mL], with a coefficient of variation of 21.9% (Benner et al., 1989) Thus, a serum cotinine level of 250 ng/mL corresponds to a daily intake of 20±4 mg of nicotine. It was suggested that this factor also applies to nonsmokers (Benowitz, 1996).

Benowitz (1996) has described a calculation that suggests a urine to blood ratio of 6, but cautions that the interpersonal variability described previously would contribute to variability in this ratio. There are insufficient data to estimate the interpersonal variability. The ratio predicted by Benowitz has had some experimental verification with a urine to blood cotinine ratio of 5 reported (Jarvis et al., 1984). Benowitz (1996) also reports that saliva to blood ratios are 1.1-1.4, leading to an essentially interchangeable use of saliva and serum cotinine data. These conversions have been summarized in **Table 3**.

Using the serum to daily nicotine intake conversion given above, and data from a large study (Pirkle et al., 1996, Figure 2), the approximate daily intake for nonsmoking subjects with no reported ETS exposure, reported ETS exposure and reported smokers is 0.024, 0.08, and 40 mg, respectively. Using a similar approach to a different set of data, a group of 91 smokers with mean

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Table 3. Conversion factors for cotinine.

To convert	Multiply by
Cotinine in serum to cotinine in urine ng/mL → ng/mL	5-6
Cotinine in serum to cotinine in saliva ng/mL → ng/mL	1.1-1.4
Cotinine in serum to daily (24 hr) nicotine intake ng/mL → mg/day	0.08±0.18

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saliva cotinine concentrations of 261.1 (±116.2) ng/mL would convert to a daily intake of 20.9 (±3.8) mg of nicotine, which may be compared to the experimentally determined 19.8 (±6.3) (Andersson et al., 1997). Other examples reinforce the evidence that serum or saliva cotinine levels can be used to estimate the mean values of nicotine intake, but the variance about those estimates for individuals may be significant, as described below. Furthermore, most of the available data are based on single samples from each individual, and very little is know about intra-individual variation.

Use of Cotinine as a Nicotine Biomarker in Exposure Studies

In a large study by Jenkins et al. (1996), saliva samples for cotinine measurements were taken for nonsmoker subjects in 16 cities in the United States the day before and the day after personal monitors were used to measure ETS parameters, including nicotine, in workplace and non-workplace environments. Four cell types were defined depending on smoking taking place away from work or in the workplace. A strong correlation ($R^2 = 0.991$) was found between the median 24-hr time weighted average (TWA) nicotine exposure and the median average salivary cotinine level on a cell-by-cell basis. Salivary cotinine levels were not well correlated with individual 24-hr TWA nicotine levels (R^2=0.105). Based on an earlier study discussed above (Saracci and Riboli, 1989), nonsmokers not exposed to ETS had an apparent estimated daily intake of 0.024 mg of nicotine, and nonsmokers exposed to ETS 0.08 mg. These compare favorably with similar calculations using the data of the study by Jenkins et al. (1996). For the cell with nonsmoking home and nonsmoking workplace, the calculated daily intake is 0.013 mg (vs. 0.024), whereas, for the subjects in the cell with smoking away from work but not at work, an estimated daily intake of 0.07

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mg (vs. 0.08) is calculated. In the most extreme case of ETS exposure both at work and away from work, a daily nicotine intake of 0.16 mg is estimated. These results appear to be consistent findings, although the Jenkins data were less so when salivary cotinine levels were below 2 ng/mL. All comparisons are based on mean or median values and may not be valid for individuals.

Although salivary cotinine measurements have been used as a biomarker for nicotine exposure for means (or medians) of large numbers of subjects from within given exposure groups, a wide range of levels of salivary cotinine is found for individual smokers and nonsmokers alike that does not correlate well to their nicotine exposure levels (Saracci and Riboli, 1989). Because of the consistent relationships between cotinine in saliva, serum and urine, it is concluded that none of these matrices can be reliably applied to individual subjects using a single measurement. As stated above, analytically valid measurements of cotinine in any of these matrices can distinguish a smoker of 20 cigarettes per day from a nonsmoker who is not exposed to ETS. Distinction between a light smoker and a nonsmoker exposed to high ETS levels is very problematic. Kemmeren et al. (1994) have used a statistical equation shown below, and based on expressions for "t" values, to predict the number of replicate measurements needed to estimate the "habitual" serum cotinine level of a subject within a selected percentage of the "true value." The expression used is

$$k_{0.95} = (1.96 \times CV/D)^2$$

in which "k" is the number of measurements needed to estimate the habitual cotinine level within a certain percentage "D" of the intra-personal variation "CV" at a 95% confidence level. The intra-

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personal coefficient of variation (CV) was estimated to be 16.1%, although there is little supporting evidence for this value. Using this value, one calculates that a single sample can estimate the "habitual" cotinine concentration to within approximately ±32%. Ten (10) replicate samplings would be required to obtain a measure within ±10% of the "habitual" value. The issue of the time period over which sampling and measurements were or should be made was discussed briefly by Kemmeren et al. (1994), who observed that samples should be taken over a longer period of time than just a few days.

In summary, it does not seem appropriate to debate a point value for a cutoff to distinguish smokers from nonsmokers based on a single measurement of salivary cotinine. In this case, the intra-personal variation in the concentration will contribute significantly to the cotinine level, and less is known about the interpersonal variation with a fixed uptake of nicotine. These pharmacokinetic and lifestyle factors must be given more consideration when attempting to use a point value for decision making.

Salivary Cotinine Concentration Related to Air Quality Measurements

Benowitz (1996) has discussed relationships between cotinine measurements and nicotine intake. The relationships proposed have been described earlier and provide some opportunity to assess nicotine intake on a daily average. Three ETS exposure studies involving subjects from 16 cities in the United States (Jenkins et al., 1996), Stockholm, which has exceptionally low ETS (nicotine) levels (Phillips et al., 1996), and Barcelona, which tends to have

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relatively high levels of ETS both in the workplace and at home (Phillips et al., 1997), provide an opportunity to consider such relationships. In all three studies, salivary cotinine measurements were made using the RIA method both prior to and after the subjects' breathing zones were sampled using personal monitors. In the case of working subjects, dual personal monitors were used for workplace and away from workplace environments. The data obtained from the monitor samples were used to calculate 24-hour time weighted average (TWA) nicotine concentrations.

Figure 1 shows a plot of the median salivary cotinine concentration versus the 24-hr TWA nicotine in air concentration for different subject categories for all three studies. The median was used because at very low levels near analytical detection limits it is generally a more representative value than the mean. In most cases, at higher concentrations the mean and median were relatively close in value. One clear outlier point is observed in the results shown in **Figure 1**. This point represents non-working housewives or househusbands living in a nonsmoking home environment in Stockholm (Phillips et al., 1996). That datum was not included in the regression. No explanation was provided for this result, which represents by far the highest nicotine and cotinine levels found in the Stockholm study, and is almost 10-fold higher than that found for homes of workers with smoking environments. Only nine (9) subjects were in this group, and it is possible that the values obtained were not representative. There could also have been unknown extraneous sources of nicotine.

After removing the datum discussed above, the remaining data shown in **Figure 1** yield the following linear regression.

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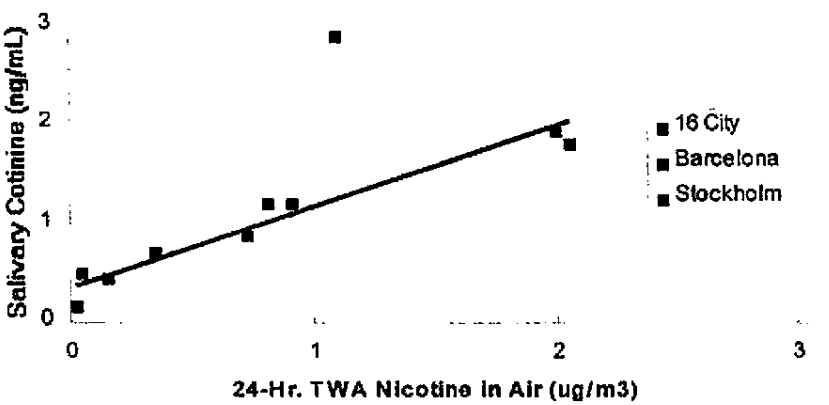

Figure 1. Plot of Salivary Cotinine Concentration versus 24-hr. TWA Nicotine Concentration Exposure in Nonsmokers

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Salivary Cotinine (ng/mL) = 0.76(±0.07)*Nicotine ($\mu g/m^3$) + 0.38(±0.07)

For both the coefficient and intercept, $p<0.001$. However, again it should be emphasized that this correlation is for median values of groups of subjects ranging from as few as 9 to over 100. Individual variation is expected to be large, but the data were not made available in the publications. Furthermore, geographic location appears to play a significant role (Jenkins et al., 1996; Phillips et al., 1996; Phillips et al., 1997). Thus, there is no evidence that salivary cotinine concentration can be used to estimate ETS exposure for an individual using such a correlation.

The intercept in the regression of the data shown in **Figure 1** is different from zero with statistical significance. The implication of this intercept is that the mean salivary nicotine concentration in all groups has a base level that is not derived from nicotine in ETS. Using the factor shown in **Table 3**, a daily intake of 30 μg/24 hr from an additional source is estimated. It is tempting to suggest that there are sources of cotinine in saliva of nonsmokers other than that from ETS exposure. Dietary sources have been suggested (Castro and Monji, 1986; Sheen, 1988; Davis et al., 1991; Domino et al., 1993; Domino, 1995). There are still open questions concerning potential cross-reactivity and other issues related to the RIA method of analysis, so that the potential for an artifact cannot be ruled out.

A factor that contributes to the intercept described above is the data analysis policy used. In the indoor air studies mentioned above (Jenkins et al., 1996; Phillips et al., 1996; Phillips et al., 1997), if the concentrations of salivary cotinine were found to be below the limit of

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quantification (LOQ) or limit of detection (LLD), the values were empirically set equal to one-half of the LLD or LOQ. Depending on a number of factors and the criteria used to establish the LLD or LOQ, this means that in some cases up 60% of the data was set to 0.5 ng/mL for salivary cotinine. With so few data points, a linear regression with an intercept near 0.5 ng/mL appears reasonable. However, one does not know what the values may be if they are below the LLD or LOQ. The analytical method used by Pirkle et al. (Pirkle et al., 1996; Bernert et al., 1997) has a reported detection limit of 50 ng/L (0.05 ng/mL). Based on estimates calculated from data shown in Figure 2 in Pirkle et al. (1996), approximately 50 % of the data for nonsmokers is found in the region below 0.5 ng/mL, further bringing into question the data treatment used in exposure studies.

Other Factors that Affect Cotinine Concentration in Biological Fluids

From the data discussed so far, it appears that salivary and serum cotinine concentrations may be correlated to exposure to nicotine in ETS for nonsmokers. Almost all exposure studies have been conducted with mature adults >17 years of age. However, a number of studies have suggested that cotinine levels for a given exposure are higher in children and blacks (Zuccaro et al., 1997; Wagenknecht et al., 1993; Pattishall et al., 1985). In one of the few studies to attempt to use multivariate methods of analysis of exposure data, Pirkle et al. (1996) performed multiple regression of log serum cotinine concentration versus such parameters as age, ethnic background, number of smokers in the house, size of household, number of rooms in the house, etc. Dietary intake was considered in the form of consumption of bell peppers and found to be of

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marginal statistical significance ($0.001<p<0.05$) in children 4-11 years of age, but not significant in adults. The regression coefficients that were obtained had a wide range of statistical reliability.

As an illustrative example, **Figure 2** shows a plot of serum cotinine concentrations calculated using regression coefficients from Pirkle versus age for white and black children living in a 5-room house, with 3 members in the household, with 1 smoker, and after consuming 20 g of bell pepper. There are two observations from **Figure 2**: all other factors being equal, young children show cotinine levels higher than adolescents, and black children have higher cotinine levels than whites. The statistical significance of many of the regression coefficients was marginal, but a trend is suggested.

In a 1984 report, Greenberg et al. determined urinary and salivary cotinine levels in infants and reported urinary cotinine to be the most reliable indicator of exposure. Median levels of cotinine in urine were 351 ng and 4 ng of cotinine/mg creatinine for ETS exposed and not exposed, respectively. From these data, Van Vunakis et al. (1987) have estimated that on a creatinine basis, urinary cotinine levels in infants are approximately 60 times greater than those found in adult males. Based on urine volume, the concentration of cotinine in the urine of infants is 8 and 1.6 times as much as the ETS-exposed and highly exposed groups, respectively. Weaver et al. (1996) determined the urinary cotinine levels of 79 inner-city children and found a mean of 54.7 ± 45.6 ng/mL with a range of 1-244 ng/mL.

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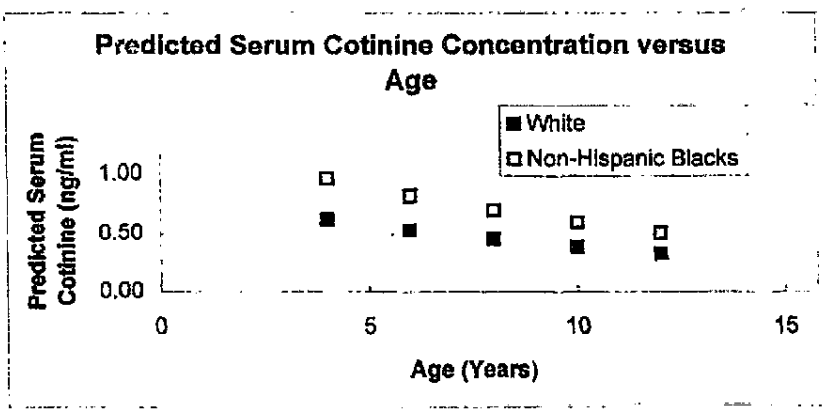

Figure 2. Use of Multivariate Analysis of NHANES III" Data to Illustrate the Effect of Age and Ethnicity
(NHANES III = Third National Health and Nutrition Examination Survey)

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Clearly, the age and ethnic background of young children must be taken into account when the cotinine levels of this group are used to infer levels of exposure to ETS. If this factor is not taken into consideration, cotinine levels found in exposure studies can be misinterpreted as excessive exposure.

Conclusions

Cotinine is not a perfect biomarker for nicotine exposure. Issues such as interpersonal variation in metabolism, non-tobacco sources of nicotine, and how well nicotine represents ETS exposure plague all biomarkers. For the present, cotinine appears to be the most reliable biomarker for estimating day-to-day exposure to tobacco smoke provided that it is applied only to mean (or median) values of large numbers of subjects.

There is much room to improve upon the use of nicotine metabolites as biomarkers for nicotine intake. Cotinine in serum and saliva appear to be equally advantageous over the use of urine samples; principally because of the limited number of analytically significant metabolites in these media. This is especially true if RIA methods are used, because of the potential for cross-reactivity of metabolites other than cotinine. Further, because cotinine represents 10-15% of the total nicotine metabolite in urine, variation in the larger concentrations of the other metabolites could have significant impact on the use of cotinine only in relating the result to nicotine intake.

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A viable analytical method that has a detection limit much below that of RIA and gas chromatography is needed. The method must be rugged and capable of processing large numbers of samples economically. Such a method has been developed for cotinine in serum based on liquid chromatography combined with atmospheric pressure ionization tandem mass spectrometry (LC/API/MS/MS) (Wagenknecht et al., 1993). Work is underway to apply the method to saliva samples. Initial capital outlay is high, but the ruggedness and throughput capabilities of the method are excellent.

However, saliva and serum have their own limitation as a sample matrix. Only intensive or concentration measurements may be made. To obtain data on the equivalent of nicotine eliminated over a period of time, total nicotine metabolite excretion by collecting urine samples has a number of advantages. **Table 2** shows that approximately 90% of the total nicotine metabolite in urine could potentially be estimated by that approach. Therefore, a method using enzymatic hydrolysis of the glucuronide conjugates of nicotine and its metabolites in urine, with the subsequent determination of nicotine, cotinine and *trans*-3-hydroxycotinine is required. This method should have a detection limit to permit quantitative data to be obtained for subjects with the lowest level of nicotine exposure. It is likely that modification of existing procedures coupled with LC/API/MS/MS could meet these needs.

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What Is Philip Morris Doing About Cotinine

Until an even more appropriate biomarker for nicotine intake is found, cotinine is currently the one of choice. The issue is not cotinine *per se*, but the limitations and misinterpretations associated with measurements and data. Several factors need to be considered. Some of these are:

1. Which biological fluid is overall the most appropriate as the measurement medium?

2. What are the recommended procedures and protocols for the acquisition, transportation, preservation and storage of these samples?

3. Which nicotine metabolites should one measure to obtain improved and appropriate accuracy in the estimation of nicotine intake?

4. What analytical method is best suited to the task?

5. How should data be analyzed?

6. How can the proliferation of expedient but questionable analytical practices be contained?

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A number of these issues are being addressed directly or indirectly by Philip Morris-supported programs.

1. *Which biological fluid is overall the most appropriate as the measurement medium?*

Each of the principal biological fluids used for biomarker measurements, serum, saliva and urine, has advantages and disadvantages as described above. The analytical methods that may be applicable also have a range of strong and weak points. In 1986, a group of experts met to discuss these issues in detail (Watts et al., 1990). This group concluded that "estimation based on urinary cotinine excretion would be less reliable than estimation based on plasma or salivary levels." They also found that "good correlations were reported between saliva and blood for results from the same subject," as found by others (Curvall et al., 1989). The number of nicotine metabolites in urine, the interpersonal variation, and the issues of using the CCR method that result from creatinine concentration variation, all counter the relative convenience of collecting urine samples.

Following the recommendations given above, Philip Morris is participating in a program to carefully investigate the use of saliva as the sample medium of choice. Although urine has considerable interest from a pharmacological perspective, as a sample medium used strictly to estimate nicotine exposure, either serum or saliva appears to have the advantage. Work is underway to establish appropriate sampling protocols and analytical methodologies for

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saliva. An excellent method for cotinine determinations in serum was recently published (Bernert et al., 1997).

2. *What are the recommended procedures and protocols for the acquisition, transportation, preservation and storage of these samples?*

A number of recommendations for sample acquisition and handling for saliva have been reported. The subject is too detailed to be reviewed here. Philip Morris is participating in a study to evaluate a number of factors in the subject preparation, sample collection, sample preservation, sample transportation and storage for saliva. A method and procedures document is planned.

3. *Which nicotine metabolites should one measure to obtain improved and appropriate accuracy in the estimation of nicotine intake?*

For a variety of reasons, the recommendations reported above (Watts et al., 1990) have not been adopted by many researchers who conduct epidemiological investigations, particularly with respect to the use of urine as the sample medium. If nicotine metabolites are to be used in urine, improved analytical methods are required for acceptable results because of the number and variation in the nicotine metabolites. Philip Morris is conducting a statistical analysis of existing data to address this issue. The outcome of this analysis will be an

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opportunity to define the analytical needs to minimize variations resulting from the complexities of a mixture of metabolites.

4. *What analytical method is best suited to the task?*

Philip Morris is participating in the development of an analytical method for cotinine and possibly *trans*-3-hydroxycotinine in saliva. For cotinine at least, the lower limit of quantification (LOQ) is expected to be approximately 0.05 ng/mL using conservative statistical policies. This may be compared with the current approximately 0.5-1.0 ng/mL using RIA methods. The method will have relatively high initial capital costs, but is capable of a throughput of over 100 samples per day. Under consideration is the participation in the development of an alternative method for urine that will involve enzymatic hydrolysis of the glucuronide conjugates of nicotine, cotinine and *trans*-3-hydroxycotinine with the subsequent determination of these three metabolites.

5. *How should data be analyzed?*

There are two issues with data analysis relative to cotinine that are present in the literature.

a.) In exposure studies, cotinine levels below the limit of quantification (LOQ) of the analytical method used are empirically set equal to 50% of the LOQ. This has the negative impact that regardless of how much ETS exposure is reduced, a finite

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cotinine level is guaranteed. This can result in a misuse of values below an LOQ. As ETS exposure is reduced, new analytical methods are required to demonstrate reduction in nicotine intake.

b.) Smoker-nonsmoker discrimination is based on the use of a point value "cut-off" approach. This assumes that anyone below a set value of cotinine level is a nonsmoker, and anyone above that value is a smoker. This is not consistent with biological diversity. New methods of distributive data analysis that permit incorporation of a variety of factors to predict the probability of a single result are needed. Philip Morris is participating in the evaluation of such methods of data analysis.

6. *How can the proliferation of expedient but questionable analytical practices be contained?*

For a variety of reasons, often commercial, new quick and easy methods for the detection and alleged quantification of cotinine or other biomarker for nicotine appear in the literature, in patents or in the marketplace. These devices offer quick, simple and near-patient determinations that may be used for various purposes, such as establishment of smoking status for insurance applications. The complexities described above all but preclude successful use of such simple, nonselective "dip-stick" tests. One such device is known as NicCheck™ on the market and is FDA approved for physician use for detection of smokers.

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Philip Morris is participating in an investigation of this device to ascertain its reliability, especially against potential false positive results.

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Appendices

The following papers are appended to this section:

Schepers, G., Demetriou, D., Rustemeier, K., Voncken, P., and Diehl, B., Nicotine phase 2 metabolites in human urine -- structure of metabolically formed *trans*-3'-hydroxycotinine glucuronide, *Med. Sci Res.* 20: 863-865 (1992). **[Tab A]**

Schepers, G., Rustemeier, K., Walk, R.A., and Hackenberg, U., Metabolism of S-nicotine in noninduced and Aroclor-induced rats, *Eur. J. Drug Metab. and Pharmacokin.* 18: 187-197 (1993). **[Tab B]**

Schepers, G., and Walk, R.A., Cotinine determination by immunoassays may be influenced by other nicotine metabolites, *Arch. Toxicol.* 62: 395-397 (1988). **[Tab C]**

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Med. Sci. Res., 1992; 20, 863–865

Nicotine phase 2 metabolites in human urine – structure of metabolically formed *trans*-3′-hydroxycotinine glucuronide

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Keywords: Urine, nicotine, cotinine, *trans*-3′-hydroxycotinine, glucuronic acid conjugates, sulphuric acid conjugates, TSP-MS, NMR.

Introduction: Evidence for phase 2 metabolites as major urinary nicotine metabolites in humans has recently been presented by several authors [*e.g.*, 1–4]: conjugates of nicotine, cotinine, and 3′-hydroxycotinine were quantified in urine samples indirectly by measuring the increase in the amounts of nicotine, cotinine, and 3′-hydroxycotinine after enzymatic hydrolysis using β-glucuronidase. This led to the suggestion that nicotine phase 2 metabolites are glucuronides of nicotine and its respective phase 1 metabolites.

Caldwell *et al.* [5] recently carried out thermospray liquid chromatography/mass spectrometry (MS) of a smoker's urine and electrospray MS of a high performance liquid chromatography (HPLC) fraction of it. The retention time and mass spectra were compared with those obtained for the fully synthesised and spectrometrically characterised reference substance. They claimed that N-β-D-glucopyranuronosyl-(S)-(–)-cotininium inner salt is a major urinary nicotine phase 2 metabolite.

In a very recent study [6], we analysed a smoker's urine by HPLC after derivatisation with 1,3-diethyl-2-thiobarbituric acid (DETBA), which is selective for compounds containing unsubstituted pyridine nitrogen: in addition to large peaks derived from *trans*-3′-hydroxycotinine (THOC), nicotine (NIC), and cotinine (COT), a large peak representing nicotine phase 2 metabolites was found. This peak was not detectable in the DETBA assay when the urine was treated with an enzyme preparation containing β-glucuronidase and sulphatase. The products of the enzyme cleavage were mainly THOC (67%), COT (31%) and a small amount of NIC (2%).

The objective of the present study was to determine which substituents are conjugated to the nicotine phase 1 metabolites and at which position.

Materials and methods: CH_2Cl_2 was from Merck (Darmstadt, Germany), DETBA from Aldrich (Steinheim, Germany), S-nicotine, chloramine-T, chloramphenicol β-D-glucuronide, β-glucuronidase (*Helix pomatia* type HP-2) and arylsulphatase (*Helix pomatia*, type H-2) from the Sigma Chemical Co. (Deisenhofen, Germany), COT from Roth (Karlsruhe, Germany), and THOC from Dr G. Neurath (Institut für Biopharmazeutische Mikroanalytik, Hamburg, Germany).

The pooled human urine sample was randomly collected (male Caucasian smoker, approximate daily consumption 50 cigarettes, nicotine delivery 1 mg per cigarette). In order to remove most of the nicotine phase 1 metabolites, aliquots of the urine were alkalised by NH_4OH to *p*H 10.8 and extensively washed with CH_2Cl_2 (4 times, twice the sample volume). Hydrolysis of the CH_2Cl_2-washed urine sample was performed enzymatically and by acid and alkaline treatment. The conditions are given in Table 1. The nicotine metabolites before and after hydrolysis were analysed using the DETBA HPLC assay [6].

Partial purification of nicotine phase 2 metabolites for MS and NMR analysis was performed starting with 500 mL of the CH_2Cl_2-washed urine. The purification procedures consisted of size-exclusion chromatography on Sephadex G10 (Pharmacia, Freiburg, Germany), collection of the fractions representing the first DETBA-positive peak, chromatography of these fractions by cation-exchange HPLC on a Partisphere SCX cartridge (Whatman, Maidstone, UK), repetitive chromatography of the DETBA-positive fractions by ion-pair HPLC on Radial Pak C18 cartridge connected in series with a Nova-Pak C18 column (Waters, Eschborn, Germany), and repetitive chromatography of the DETBA-positive fractions by reverse phase HPLC on the above-mentioned C18 columns. Two DETBA-positive fractions were obtained. For NMR analysis, fraction 2, representing the major DETBA-positive peak, was further purified by hydrophilic interaction liquid chromatography on a polyhydroxyethyl-aspartamide column (ICT, Frankfurt, Germany).

Thermospray mass spectrometry (TSP-MS) was performed on a mass spectrometer coupled to a TSP interface (QUATTRO, Fisons Instruments, Mainz, Germany). The solvent was a mixture of methanol and 0.1 mol L^{-1} ammonium acetate in water (50/50, v/v). The sample was directly applied by loop injection.

NMR spectrometry was performed on a Bruker AC-P 300

Table 1: Hydrolysis of CH_2Cl_2-washed smoker's urine.

Treatment	Hydrolysis condition				Metabolite detected[a]			
	Activity (units mL^{-1})	*p*H	Incubation time (h)	Temperature (°C)	C	NIC	COT	THOC
β-glucuronidase	6,000	4.7	18	37	0	+	–	+
Arylsulphatase	1,000	7.0	18	37	0	+	+	+
Alkali	–	14.0	16	70	+ (↓)	0	+	0
Acid	–	1.0	16	70	+ (↓)	+	0	+

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NMR spectrometer. ^{1}H-NMR- and H, H-COSY-90 spectra of the samples (approx. 250 µg in 0.5 mL CD_3OD) were recorded.

Results and discussion: In the washed urine, the peak representing nicotine phase 2 metabolites, designated C for conjugates in Table 1, completely disappeared after treatment with β-glucuronidase and with sulphatase but only partially decreased after acidic and alkaline hydrolysis (Table 1). The differences found between alkaline and acidic hydrolysis might indicate that COT is conjugated by different substituents and/or at different sites than THOC and NIC.

In the TSP-MS of the partially purified fraction 1 (Figure 1a), $[M + H]^+$ ions corresponding to sulphuric acid conjugates of COT (m/z 257) and THOC (m/z 273) were found. The assumption that these ions represent nicotine phase 2 metabolites is supported by the occurrence of fragments with m/z 177 (COT) and m/z 193 (THOC). Several other ions in this spectrum might represent amino acids still present in this fraction (*e.g.*, m/z 134: asparagine, m/z 150: methionine).

In the TSP-MS of fraction 2 (Figure 1b), $[M + H]^+$ ions corresponding to a glucuronide of THOC (m/z 369) and of its aglycon THOC (m/z 193) were found. The fragment at m/z 351 can be explained by the loss of water (18 Daltons).

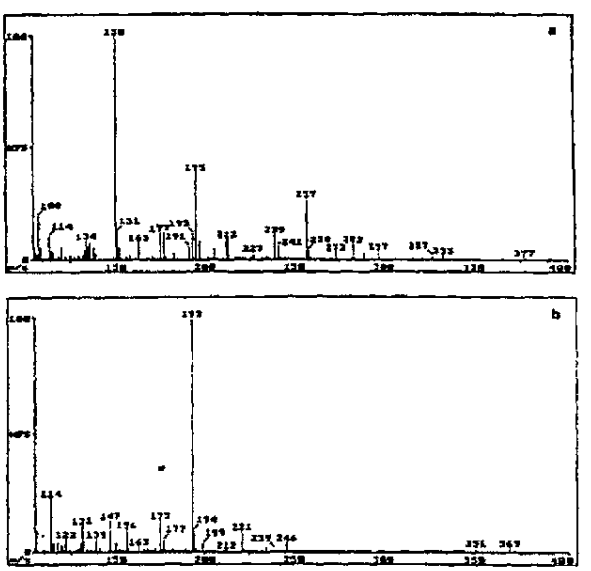

Figure 1: TPM-MS of partially purified nicotine phase 2 metabolites. a: fraction 1. b: fraction 2.

Table 2: Chemical shifts of pyridine, pyrrolidine and glucuronic acid ring protons.

H-No.	Chemical shift (ppm) THOC	Chloramphenicol-β-D-glucuronide	Fraction 2
2	8.4	–	8.4
4	7.7	–	7.7
5	7.5	–	7.5
6	8.5	–	8.5
3'	4.5	–	4.8
4'a,b	2.4, 2.3	–	2.6, 2.4
5'	4.8	–	4.8
6'	2.7	–	2.7
1"	–	4.4	4.4
2"	–	3.3	3.3
3"	–	3.4	3.4

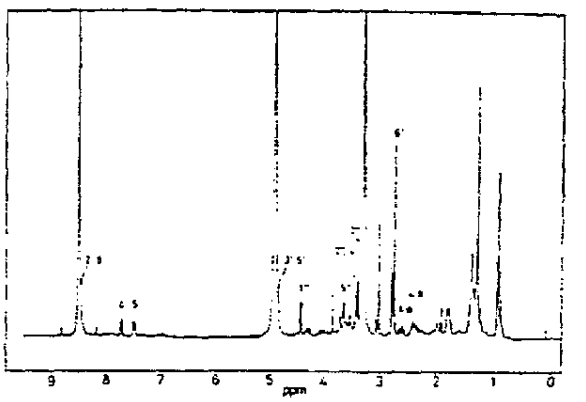

Figure 2: One-dimensional ^{1}H-NMR spectrum of partially purified nicotine phase 2 metabolites, fraction 2.

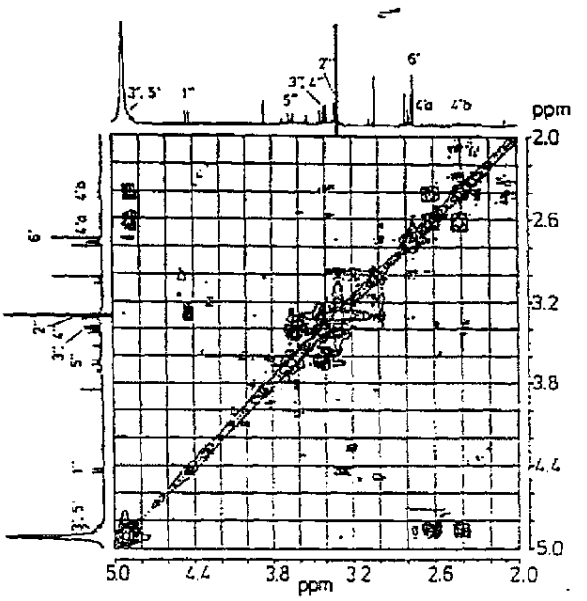

Figure 3: Two-dimensional H, H-COSY 90 spectrum of partially purified nicotine phase 2 metabolites, fraction 2.

In the one-dimensional ^{1}H-NMR spectrum (Figure 2) and in the H,H,-COSY 90 spectrum (Figure 3) of fraction 2, the signals of a substituted THOC were observed. A comparison of the chemical shifts with those of THOC (Table 2) shows that the substitution has to be at the oxygen of the hydroxyl-group at the 3'-position in the pyrrolidine ring. The ^{1}H-NMR-signals of the sugar moiety show complex unresolved multiplets.

Therefore, a straightforward analysis of this part of the spectrum was not possible. The structure of the sugar moiety, however, could be established by comparison with the spectrum of chloramphenicol-β-D-glucuronide, which showed similar chemical shifts for the glucuronic acid ring protons (Table 2). Spin simulation analysis based on the coupling constants obtained from the first order evaluation of this spectrum predicted fine structures for the multiplets of

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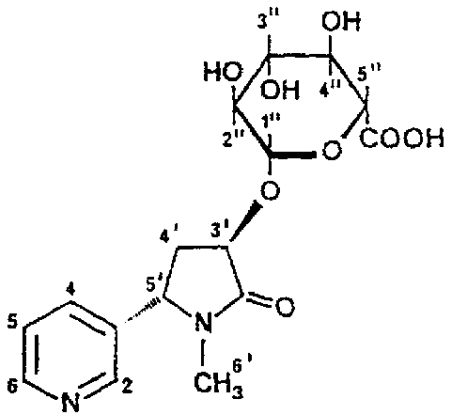
in our urine sample the major nicotine phase 2 metabolite was *trans*-3'-hydroxycotinine-O-β-D-glucuronide:



This is the first time that a metabolically formed nicotine phase 2 metabolite has been identified. Using MS and NMR spectrometry, this metabolite was found to be the O-β-D-glucuronide of THOC. In addition, evidence is given of further urinary nicotine phase 2 metabolites, *i.e.*, sulphuric acid conjugates of COT and THOC. The differences between our results and those reported in literature, but also between the published data itself concerning the quantitative results, can be explained by inter-individual variation in both nicotine phase 1 and phase 2 metabolism.

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Paper received: 10th September, 1992.

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EUROPEAN JOURNAL OF DRUG METABOLISM AND PHARMACOKINETICS, 1993, Vol. 18, No. 2, pp. 187–197

Metabolism of S-nicotine in noninduced and Aroclor-induced rats

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Received for publication: July 17, 1992

Keywords: Nicotine, urinary metabolites, excretion kinetics, Aroclor induction, sex differences, stereoselectivity

SUMMARY

The urinary excretion of nicotine and its metabolites in noninduced and Aroclor-induced male and female rats has been determined following intravenous administration of 2'-[^{14}C]-labeled S-nicotine at a dose of 4.6 μmol/kg. Complete recovery of the administered radioactivity was achieved: 95% in urine and 4% in feces over 96 h and 1% remaining in the body. More than 40 nicotine metabolites were found by radio-HPLC; 19 were identified including the *cis/trans*-diastereomers of nicotine-N'-oxide and 3'-hydroxycotinine. The urinary metabolite profile and excretion kinetics of nicotine and its metabolites were significantly different between noninduced and Aroclor-induced rats. The major urinary nicotine metabolite in the noninduced rat was *cis*-nicotine-N'-oxide. In the Aroclor-induced rat, cotinine metabolites were the major metabolites found. Sex differences were found for the urinary nicotine metabolite profile, mainly expressed in the excretion of *cis*-nicotine-N'-oxide, 29% in the male and 17% in the female noninduced rat, and the excretion of cotinine, 5% in the male and 12% in the female noninduced rat. High stereoselectivity was found for the formation of the *cis/trans*-diastereomers of nicotine-N'-oxide as well as of 3'-hydroxycotinine, the stereoselectivity being more pronounced in male rats.

INTRODUCTION

Nicotine metabolism has been extensively studied, and more than 20 nicotine metabolites resulting from 5 different metabolic pathways have been described [for review, *see* (1–5)]. The metabolism of the naturally occurring S-enantiomer and of the synthetic R-enantiomer of nicotine were shown to be different in vitro (6) as well as in vivo (7), and one enantiomer was found to interfere with the metabolism of the other (8). In the aforementioned in vivo metabolism study in the rat using enantiomerically pure nicotine tritiated at the methyl group (7), only a limited number of metabolites could be detected. This is because the methyl group is lost at early steps in the metabolic pathways. Therefore, the present study was performed using the enantiomerically pure S-nicotine, [^{14}C]-labeled at the 2'-position in the pyrrolidine ring.

It is known that the metabolism and biokinetics of nicotine are influenced by genetic (e.g. strain, sex) and exogenous factors (e.g. drug treatment, diet) [reviewed in (5)], which can affect the induction status of the metabolizing enzymes. The influence of selective inducers of various cytochrome P-450 isozymes, e.g. phenobarbital, 5,6-benzoflavone, β-naphthoflavone, 3-methylcholanthrene, or ethanol on nicotine metabolism has been shown in vitro on subcellular fractions or isolated organs [e.g. (9–15)]. In vivo, the influence of

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selective cytochrome P-450 isozyme induction on nicotine metabolism in the rat was shown using racemic nicotine (16, 17).

To our knowledge, no in vivo study with nicotine has been performed using a nonselective inducer for various enzyme systems, e.g. Aroclor 1254, a mixture of polychlorinated biphenyls commonly used for the preparation of the postmitochondrial (S9) fraction for metabolizing promutagens in in vitro assays (18). The influence of such a nonselective inducer on nicotine metabolism, however, is of special interest since nicotine metabolism includes not only P-450-related but also other enzymatic pathways.

In the present study, the in vivo metabolism and biokinetics of S-[pyrrolidine-2'-^{14}C]-nicotine as well as the influence of enzyme induction by Aroclor 1254 are presented in detail for male and female rat. In addition, a comprehensive analysis of major and minor urinary nicotine metabolites, based on the completely recovered radioactivity, is given.

MATERIALS AND METHODS

Test substance

S-[Pyrrolidine-2'-^{14}C]-nicotine-di-L-tartrate salt (^{14}C-NIC) was synthesized by Chemsyn Science Laboratories (Lenexa, Kans, USA): specific radioactivity: 58.8 mCi/mmol, chemical purity: > 97%, radiochemical purity: > 98%, optical purity: > 98%. The optical purity was confirmed by determining the enantiomeric excess by HPLC (19) and GC on chiral columns (publication in preparation). The ^{14}C-NIC was dissolved in phosphate-buffered saline (phosphate 10 mmol/l, pH 7.2, NaCl 140 mmol/l) to a final activity of 2.5 mCi/ml, and aliquots of this stock solution were stored at –80°C.

Reference compounds

S-nicotine (NIC) was from Sigma (Deisenhofen, Germany); cotinine (COT) from Roth (Karlsruhe, Germany); 3-pyridylacetic acid (PAAc) from Aldrich (Steinheim, Germany). Dihydrometanicotine (DHMNIC), *cis*-3'-hydroxycotinine (CHOC), *trans*-3'-hydroxycotinine (THOC), cotinine-N-oxide (CNO), norcotinine (NCOT), nornicotine (NNIC), 4-(3-pyridyl)-4-oxo-N-methyl-butyramide (POMBAm), 4-(3-pyridyl)-4-oxobutyric acid (POBAc), N,N'-dimethylnicotinium diiodide (NN'DMN), 4-(3-pyridyl)-4-methyl-aminobutyric acid (PMABAc), 4-(3-pyridyl)-4-aminobutyric acid (PABAc), 4-(3-pyridyl)-butyric acid (PBAc), 4-(3-pyridyl)-4-hydroxybutyric acid (PHOBAc), and 5-(3-pyridyl)-tetrahydrofuranone (PTHF) were from Dr G. Neurath (Institut für Biopharmazeutische Mikroanalytik, Hamburg, Germany). 5'-Hydroxycotinine (5HOC), demethylcotinine-N-oxide (DMCNO), and the methyl ester of POBAc were from Philip Morris (Richmond, Va, USA); 1'(R)-2'(S)-*cis*- and 1'(S)-2'(S)-*trans*-nicotine-N'-oxide (CNN'O and TNN'O) were from Prof. J. Gorrod (Kings College, London, UK); N-methylnicotinium iodide (NMNIC), N'-methylnicotinium iodide (N'MNIC), N-methylcotininium iodide (NMCOT), and N-methyl-N'-oxonicotininum iodide (NMNN'O) were from Prof. P. Crooks (University of Kentucky, Lexington, USA). For the structural formulae of nicotine metabolites and reference compounds, *see* Figure 1.

Other chemicals

Aroclor 1254 was from Analabs (via Antechnica, Etlingen, Germany) and was dissolved in corn oil (200 g/l). All other chemicals were from commercial sources and of the highest purity available.

Animals

Male and female Sprague Dawley CrL:CD(SD)BR outbred albino rats (Charles River Wiga, Sulzfeld, Germany) were used. 14 days before administration of ^{14}C-NIC, a vascular access port (Implantofix-R, Braun Melsungen, Melsungen, Germany) was subcutaneously implanted with access to the vena jugularis (20). The rats were individually housed in polycarbonate cages with bedding material. During the whole study, water and diet (HERILAN MRH-HALTUNG, Eggersmann, Rinteln, Germany) were provided *ad libitum*. The room temperature was maintained at 22 ± 2°C. The light/dark cycle was 12:12 h, the light cycle starting at 06.00 am.

Administration of Aroclor

Those rats used to study the influence of enzyme induction on the metabolism and biokinetics of NIC received a single i.p. dose of Aroclor 1254 (0.5 g/kg) 5 days prior to ^{14}C-NIC administration. The protocol was the same as that used for the preparation of the postmitochondrial fraction for metabolizing promutagens in in vitro assays (18).

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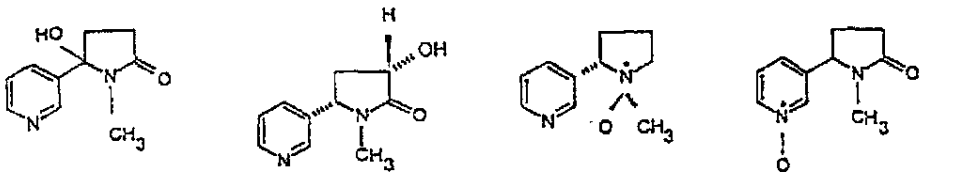
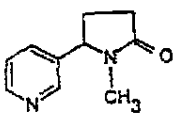


5HOC

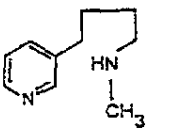
CHOC

CNN'O

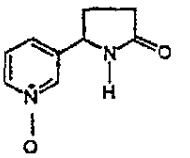
CNO



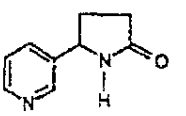
COT



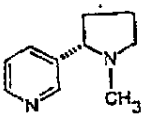
DHMNIC



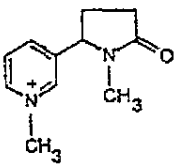
DMCNO



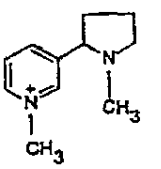
NCOT



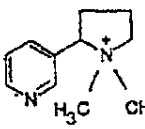
NIC



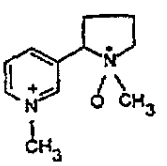
NMCOT



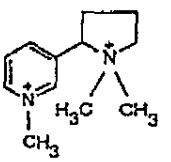
NMNIC



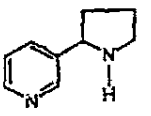
N'MNIC



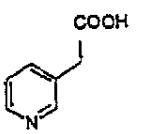
NMNN'O



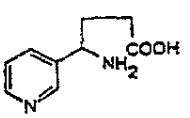
NN'DMNIC



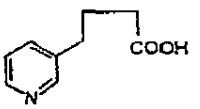
NNIC



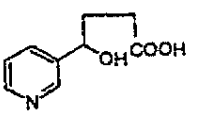
PAAc



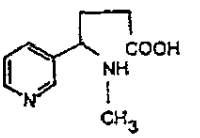
PABAc



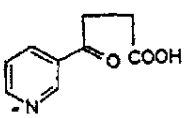
PBAc



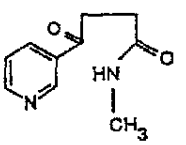
PHOBAc



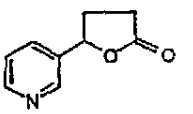
PMABAc



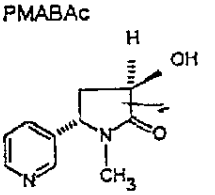
POBAc



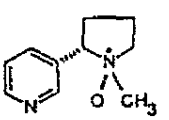
POMBAm



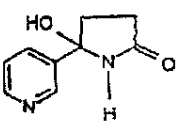
PTHF



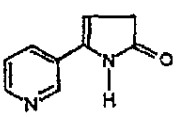
THOC



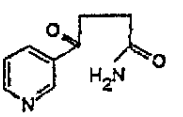
TNN'O



Allohydroxy-demethylcotinine



Demethylcotinine-$\Delta^{4',5'}$-enamine



4-(3-Pyridyl)-4-oxo-butyramide

Fig. 1 : Structural formulae of nicotine metabolites and reference compounds.

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Administration of S-nicotine

The ^{14}C-NIC stock solution was diluted with physiological saline to a final concentration of 0.9 µmol/ml. The rats (230–300 g body weight) received an i.v. dose of 4.6 µmol ^{14}C-NIC/kg (0.75 mg nicotine base/kg) at a rate of 13.3 µl/(kg· s) by a motor-driven Hamilton syringe via the implanted vascular access port. Following ^{14}C-NIC administration, the rats were housed in metabolism cages for up to 96 h.

Collection of urine and feces

Urine was collected in 50 ml ice-cooled polypropylene tubes preloaded with 200 µl of L-tartaric acid (5 mol/l). Feces were collected in ice-cooled glass containers. The surfaces of the metabolism cages were rinsed with water and the wash water collected. The urine samples were centrifuged and stored at –20 °C until chromatographic analysis.

Determination of radioactivity

Radioactivity was determined by liquid scintillation counting (LSC) using a TRI-CARB 2550 TR liquid scintillation analyzer (Canberra-Packard, Frankfurt, Germany). Urine and wash water samples were prepared directly in Aquasafe 300 (Zinsser Analytic, Frankfurt, Germany). Feces and tissue samples were homogenized and the radioactivity was determined following oxidative combustion in an OX500 sample oxidizer (Harvey Instruments, via Zinsser Analytic, Frankfurt, Germany), by trapping the CO_2 in Oxysolve C-400 (Zinsser Analytic, Frankfurt, Germany).

Radiochromatography

HPLC of the urine samples was performed using a Hewlett Packard 1090 L liquid chromatograph (Waldbronn, Germany) equipped with a ternary solvent delivery system and a diode array detector (10 µl cell) connected in series to a RAMONA 90 radioactivity detector (150 µl calcium fluoride solid scintillator flow cell, Raytest, Straubenhardt, Germany). The analog signals of the UV detector (254 nm) and of the radioactivity detector were evaluated by calculating peak areas using a chromatography data system (Multichrom, VG Instruments, Wiesbaden, Germany). Peak identification was performed by comparison with the retention times of the reference compounds. Radio-HPLC was performed by ion-pair chromatography on two analytical Nova-Pak C18 columns connected in series. A nonlinear ternary solvent program was applied. The mobile phase consisting of a potassium phosphate buffer (pH 3.6), containing pentane-, heptane-, and octane-sulfonic acid, and acetonitrile was pumped at a flow rate of 0.6 ml/min. A typical radiochromatogram is shown in Figure 2. To verify the results obtained by ion-pair chromatography, a cation-exchange HPLC method based on the method of Cundy and Crooks (21) was used following modifications. Full details of both HPLC methods are described by Demetriou et al. (22).

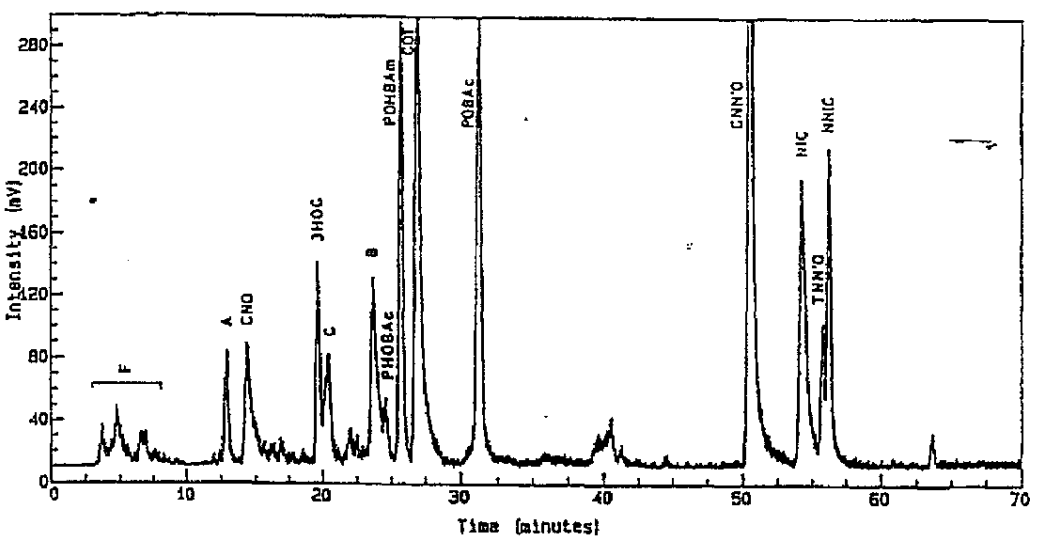

Fig. 2: Ion-pair radio-HPLC of a 24 h urine sample from a noninduced female rat dosed with 2'-[^{14}C]-labeled S-nicotine.

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Evaluation of the urinary excretion kinetics

To evaluate the urinary excretion kinetics of NIC and its major metabolites, sigma minus plot analysis was used (23): the difference between the total amount of a metabolite excreted (U_∞) and the amount excreted up to a given time point (U_t) relative to the administered NIC was plotted logarithmically versus time.

RESULTS AND DISCUSSION

Total excretion and recovery of the administered radioactivity

Rats dosed with ^{14}C-NIC excreted 95% of the administered radioactivity in urine within 96 h. The excretion in feces was 4% and the radioactivity determined in the body 1%. The exhaled radioactivity was determined to be less than 0.1%. Hence, complete recovery of the administered radioactivity was achieved. The results were the same for both noninduced and Aroclor-induced rats.

Profile of urinary metabolites

More than 40 peaks were found in the radiochromatograms of the urine samples; 18 of them coincided with chromatographic properties of authentic synthetic compounds. For the diastereomers of nicotine-N'-oxide, CNN'O and TNN'O, baseline separation was achieved with both ion-pair and ion-exchange HPLC. However, due to insufficient separations THOC and CHOC were determined in sum and designated as 3'-hydroxycotinine (3HOC). Furthermore, PMABAc and its lactam COT as well as 5HOC and its ring-opened tautomer POMBAm were not separated. They were designated as COT and POMBAm, respectively.

When considered according to the amounts excreted in the urine of noninduced male rats within 24 h following the administration of NIC, the first 20 metabolites accounted for more than 90% of the excreted radioactivity (Fig. 3). Of these 20 metabolites, 12 were identified. Unidentified NIC metabolites represented 20% of the radioactivity excreted in the urine of noninduced and 35% of the radioactivity excreted in the urine of the Aroclor-induced rats.

The major urinary NIC metabolite in the noninduced male rat is CNN'O (29%), followed by NIC (11 %) and by POBAc (10%) (Fig. 3a). COT accounted for 5% of the administered radioactivity. The NIC found may have been eliminated either unmetabolized or after metabolic formation followed by the reduction of nicotine-N'-oxide (6, 24–27). In the metabolite profile of the Aroclor-induced male rats, the formation of secondary metabolites via COT predominated, thus indicating a shift from the N- to the C-oxidation pathway. The major metabolites in the induced rat were CNO (14%), POBAc (12%), POMBAm (9%), and 3HOC (7%), whereas CNN'O decreased to 5% (Fig. 3b). COT accounted for less than 2% of the

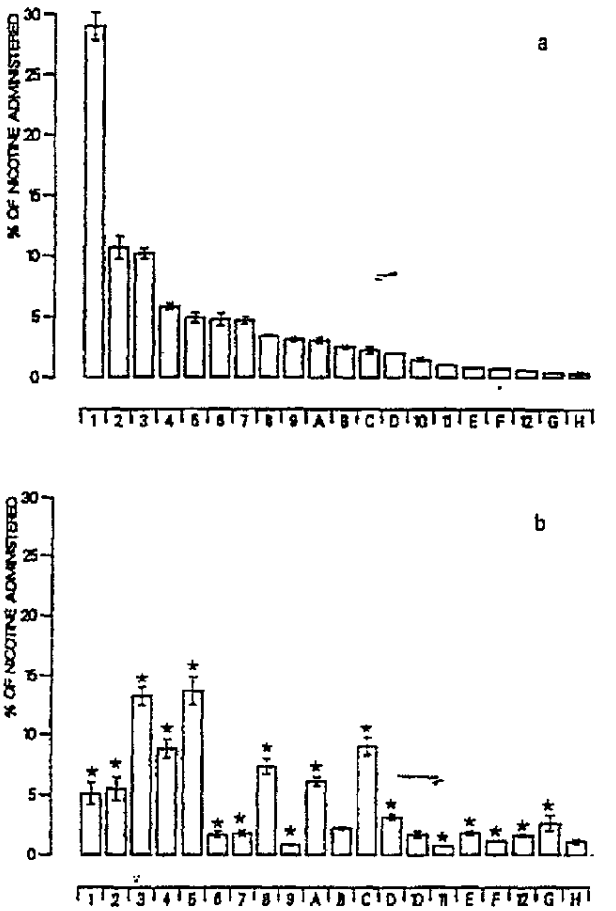

Fig. 3: Profile of urinary nicotine metabolites excreted over 24 h after i.v. S-nicotine administration to male rats. a: noninduced (M ± SE, n = 5); b: Aroclor-induced (M ± SE, n = 5). 1: CNN'O; 2: NIC; 3: POBAc; 4: POMBAm; 5: CNO; 6: COT; 7: NNIC; 8: 3HOC; 9: TNN'O; 10: PHOBAc; 11: NCOT; 12: PAAc; A to H: unknown (F: phase 2 metabolites?). Statistically significant differences ($P < 0.05$) between noninduced and Aroclor-induced rats are indicated by stars.

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administered radioactivity. For NIC and most of its metabolites, statistically significant differences were seen in the amounts excreted in the urine between noninduced and Aroclor-induced rats.

Of the metabolites not shown in Figure 3, each contributed to less than 1% of the administered radioactivity. These included the following identified metabolites NMNN'O, NN'DMNIC, PTHF, PABAc, N'MNIC, and PBAc, accounting in total for about 1% of the administered radioactivity.

A comparison of the chromatograms of the urine samples with those of the reference compounds did not indicate that DHMNIC, NMNIC, NMCOT, and DMCNO are urinary S-nicotine metabolites in the rat.

The urinary metabolite profile of R-nicotine in the rat is different from that of S-nicotine as indicated by results we have obtained in an ongoing study. Differences were pronounced, e.g. in the excretion of TNN'O, NNIC, and COT as the major R-nicotine metabolites in the noninduced rat. These results as well as the fact that S-nicotine interferes with the metabolism of R-nicotine (8) make it questionable to compare the metabolite profile obtained for S-nicotine in our study with that previously published for racemic R/S-nicotine (28).

Unidentified metabolites

Of the unidentified metabolites, those designated as B, C, and D showed different peak shapes in the chromatograms of different urine samples. This led us to the assumption that each of these peaks represents more than one metabolite. The formation of metabolite C, eluting immediately after 3HOC, was significantly increased by Aroclor induction. Metabolite A, which was also significantly enhanced by induction, is probably strongly polar as indicated by its early elution in front of CNO under both ion-pair and ion-exchange chromatographic conditions. Using ion-pair HPLC, metabolite A was found to have the same retention time as DMCNO, which has been proposed to be a nicotine metabolite (29). However, due to the different retention times of metabolite A and the reference compound DMCNO in the ion-exchange HPLC, it can be concluded that DMCNO is not a NIC metabolite in the rat.

Metabolites were observed that elute at the beginning of the chromatograms (designated together as F). This indicates that they are even more polar than metabolite A. Therefore, we assumed that they represent the nicotine phase 2 metabolites which have been found in human urine (30–35) as well as in the urine of NIC-treated rats and hamsters (34).

Recently, allohydroxydemethylcotinine has been described as a nicotine metabolite found in the urine of rats (36), and its dehydration product demethylcotinine-$\Delta^{4',5'}$-enamine in the urine of humans (32). In order to check whether these metabolites occur in the urine of NIC-treated rats, we performed a synthesis of allohydroxydemethylcotinine according to Kyeremateu by ammonolysis of the methyl ester of POBAc. Ion-pair HPLC of the reaction mixture yielded 2 major product peaks. GC/MS analysis of each of these 2 HPLC fractions yielded similar results: for each fraction 2 peaks were found by GC, one showing the mass spectrum identical with that of 4-(3-pyridyl)-4-oxobutyramide (37), the other a mass spectrum most probably of demethylcotinine-$\Delta^{4',5'}$-enamine. 4-(3-Pyridyl)-4-oxobutyramide is the ring-opened tautomer of allohydroxydemethylcotinine and therefore – analogous to 5HOC and POMBAm – we do not expect that our HPLC method is able to separate these 2 compounds. Demethylcotinine-$\Delta^{4',5'}$-enamine is the dehydration product of these 2 compounds. However, neither of the 2 product peaks in the HPLC corresponded to any of the peaks found in the radiochromatograms of the rat urine samples. Therefore, we conclude that neither allohydroxydemethylcotinine, 4-(3-pyridyl)-4-oxobutyramide, nor demethylcotinine-$\Delta^{4',5'}$-enamine are urinary metabolites of NIC in the rat.

Kinetics of urinary excretion

The sigma minus plots of NIC and its major urinary metabolites (Fig. 4) are not linear over the full time range but have three different curve shapes. The concave curves of NIC and CNN'O are linear during the first 6 h. For this period, the urinary excretion half-times ($t_{1/2}$) were calculated to be 1.6 h for NIC and 2.1 h for CNN'O in the noninduced and 0.9 and 1.3 h, respectively, in the Aroclor-induced male rats. For the other urinary metabolites in Figure 4, convex curves in the noninduced and sigmoidal curves in the induced rats were observed. This was the case not only for the secondary metabolites via COT (POBAc, POMBAm, CNO, and 3HOC) and the unidentified metabolite A but also for COT and NNIC. Both COT and NNIC are formed in a two-step reaction via iminium ion intermediates (38, 39) in contrast to CNN'O which is formed by a one-step oxidation of NIC. The fact that the excretion kinetics of the unidentified metabolite A are very similar to those of the secondary metabolites via COT and in addition that its formation is enhanced by Aroclor induction in the same way as the second-

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ary metabolites via COT led us to the assumption that metabolite A might be also a secondary metabolite via COT.

The values for the shortest excretion half-time, calculated from the steepest slope in the sigma-minus plots, are shown in Table I. The convex sigma minus

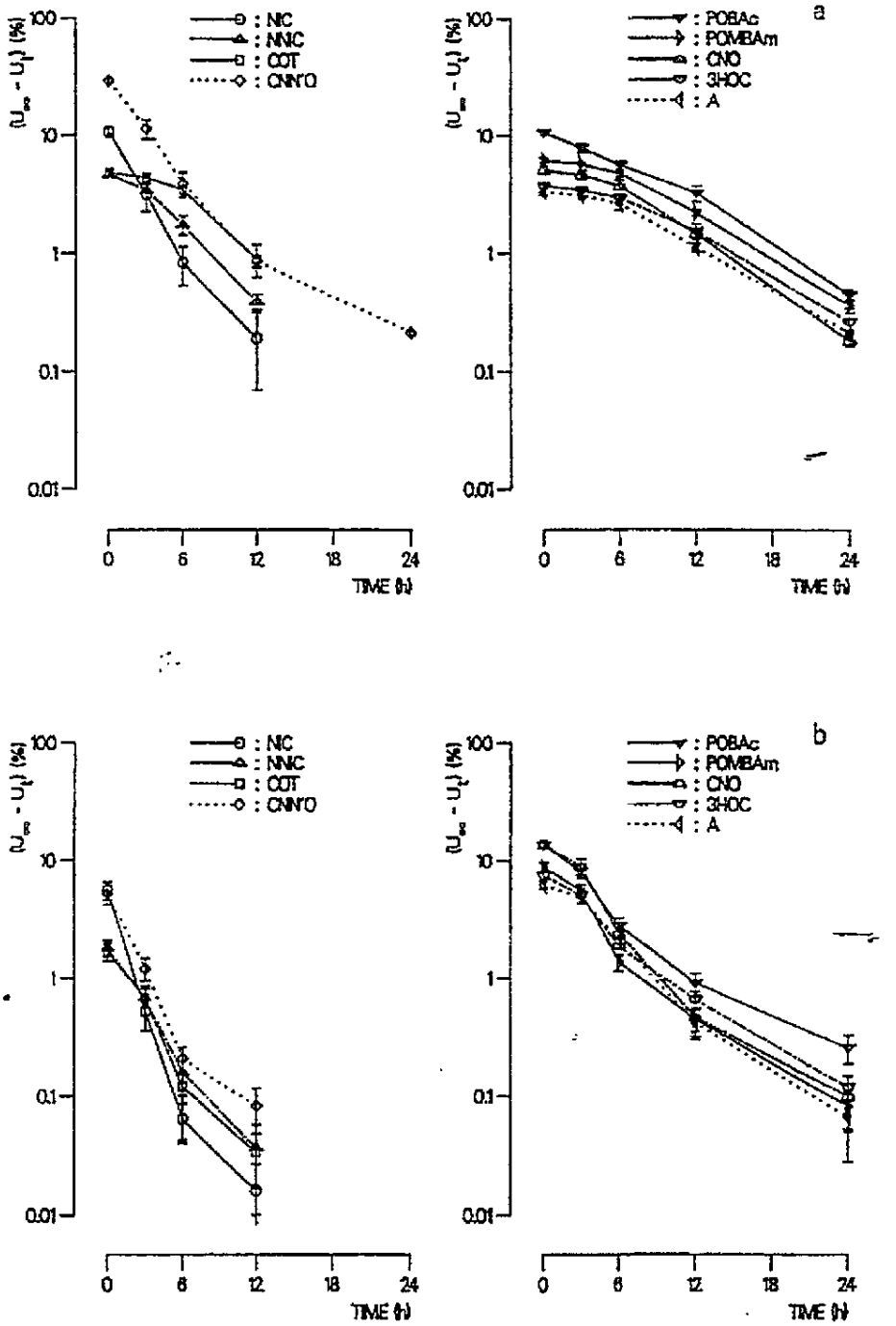

Fig 4: Sigma-minus plots of the urinary excretion kinetics of S-nicotine and metabolites in the male rat. a: noninduced (M ± SE, n = 5); b: Aroclor-induced (M ± SE, n = 5).

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Table I: Shortest excretion half-time $t_{1/2}$ of S-nicotine and metabolites in male rats.

Metabolite	*$t_{1/2}$ (h)*	
	Non-induced	*Aroclor induced*
NIC	1.6	0.9
CNN'O	2.1	1.3
NNIC	2.7	1.5
COT	3.1	1.2
POBAc	4.3	1.9
POMBAm	4.8	1.5
CNO	4.0	1.5
3HOC	4.9	2.0
A	5.0	2.6

Calculated from the steepest slope in the sigma-minus plots.

plots can be explained in that the formation rate is more rapid than the elimination rate within the first 3 h. For CNN'O, this period might be missed due to the very fast metabolism of NIC to this primary metabolite and the fast excretion kinetics of CNN'O.

Comparison of the metabolite profiles of male and female rats

In general, NIC metabolism and its inducibility were similar for male and female rats. All urinary metabolites found in the male rat were also found in the female rat. For the female as for the male rat, unidentified metabolites accounted for approx. 20% of the urinary excreted radioactivity in the noninduced and for 35% in the Aroclor-induced rat. Statistically significant differences in the excreted amount of NIC and its metabolites were found between the noninduced and induced female rats (Fig. 5): Aroclor induction leads to a reduction in the formation of CNN'O and to an enhanced formation of secondary metabolites via COT, e.g. POBAc, POMBAm, CNO, and 3HOC, as it was also found for the male rat.

For some metabolites, however, different urinary excretion was observed between male and female rats. The ratios for the excretion over 24 h and the results of the statistical evaluation of these differences are given in Table II. Major differences were seen between the noninduced male and female rats. The urinary excretion of CNN'O, which for both sexes is the major NIC metabolite, was lower and the excretion of COT was higher in the female rat. The lower N'-oxidation of nicotine observed in the female rats is in accordance with the recently reported sex differences in flavin-containing monooxygenase activity (40). Furthermore, noninduced and Aroclor-induced female rats excrete significantly higher amounts of the unknown metabolites F, which are assumed to represent phase 2 metabolites of NIC, than the male rat.

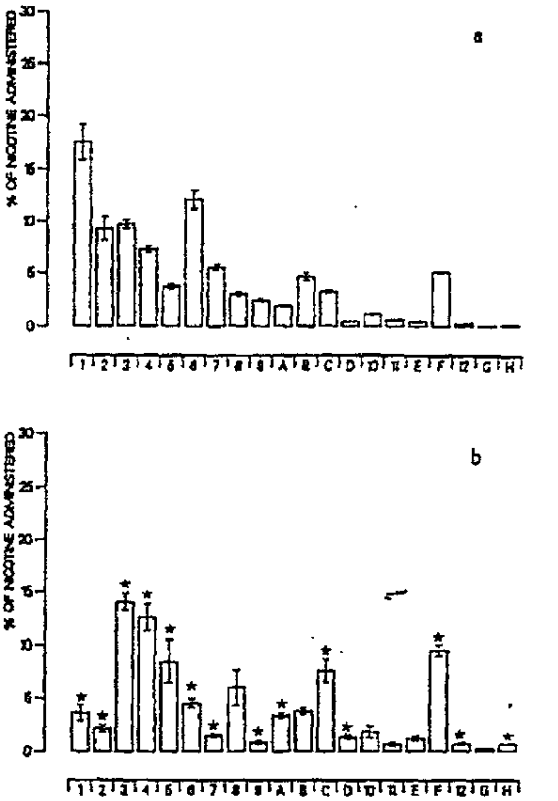

Fig. 5: Profile of urinary nicotine metabolites excreted over 24 h after i.v. S-nicotine administration to female rats. a: noninduced (M ± SE, n = 5); b: Aroclor-induced (M ± SE, n = 4). 1: CNN'O; 2: NIC; 3: POBAc; 4: POMBAm; 5: CNO; 6: COT; 7: NNIC; 8: 3HOC; 9: TNN'O; 10: PHOBAc; 11: NCOT; 12: PAAc; A to H: unknown (F: phase 2 metabolites?). Statistically significant differences ($P < 0.05$) between noninduced and Aroclor-induced rats are indicated by stars.

Stereoselectivity of S-nicotine metabolism

During the formation of nicotine-N'-oxide (NN'O) and 3HOC, two of the major NIC metabolites in the rat, a second stereogenic center in their molecular structure is formed. Therefore, it can be expected that the me-

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Table II : Ratio of urinary excretion of S-nicotine and metabolites over 24 h between male and female rats.

Metabolite	*Ratio*	
	Non-induced	*Aroclor induced*
CNN'O	1.7*(a)	1.4
NIC	1.2	2.6*
POBAc	1.0	0.9
POMBAm	0.8*	0.7
CNO	1.3*	1.6
COT	0.4*	0.4*
NNIC	0.8	1.2
3HOC	1.1	1.2
TNN'O	1.3	1.0
A	1.5*	1.8*
B	0.5*	0.6*
C	0.7	1.2
D	4.3*	2.3*
PHOBAc	1.2	0.9
NCOT	1.5*	1.1
E	1.8*	1.5
F	0.1*	0.1*
PAAc	1.9	2.3*
G	2.8*	10.0*
H	1.4	1.7

(a) Statistically significant differences ($P < 0.05$) in the urinary excretion of nicotine metabolites between noninduced male and female rats as well as between Aroclor-induced male and female rats are indicated by asterisks.

tabolism of NIC may lead to the predominant formation of one of the diastereomers of these metabolites.

We found that the in vivo N'-oxidation of NIC in both male and female rats leads predominantly to the formation of the *cis*-diastereomer of NN'O, CNN'O (*see* Table III). This stereoselectivity was statistically significantly more pronounced in the noninduced compared to the Aroclor-induced rats. This highly stereoselective N-oxidation pathway is catalyzed by flavin-containing monooxygenases (41) and has been previously reported for several in vitro studies (6, 42–44).

As already mentioned, separation of the diastereomers of 3HOC was not achieved by the radiochromatographic methods. However, we achieved their separation by HPLC after diethylthiobarbituric acid derivatization (34), and we were able to show that in both male and female rats the *trans*-diastereomer of 3HOC, THOC, is predominantly formed (*see* Table III). This confirms recently reported results (45). The stereoselectivity of the 3'-hydroxylation of COT was statistically significantly higher in the Aroclor-induced compared to the non-induced rats. The stereoselective formation of diastereomers of NN'O as well as of 3HOC was more pronounced in male than in female rats. The differences in stereoselectivity found for the formation of NN'O as well as of 3HOC in the noninduced and Aroclor-induced rats lead to the assumption that different isozymes of the respective enzymes are involved and that these isozymes are induced and/or repressed to a different extent.

CONCLUSION

The results of this study demonstrate that there are considerably more S-nicotine metabolites present in the urine of rats than previously described. Important differences exist in the metabolism and excretion kinetics between noninduced and Aroclor-induced rats and in the urinary excretion of some S-nicotine metabolites between male and female rats. The formation of the *cis/trans*-diastereomers of nicotine-N'-oxide and 3'-hydroxycotinine is highly stereoselective. The results underline the importance of exogenous factors and genetic composition on the complex metabolism and biokinetics of S-nicotine.

ACKNOWLEDGEMENTS

We thank Prof. J. Gorrod and Prof. P. Crooks for the gift of reference compounds, Dr P. Voncken for helpful discussions and for providing the GC-MS analysis, Mr D. Hanselmann for critical reading of the manuscript, and Mrs G. Fleger, Mrs G. Kunz, Mr J. Doll as well as Mr D. Demetriou for their excellent technical assistance.

Table III : Ratio of the urinary 24 h excretion of the *cis/trans*-diastereomers of nicotine-N'-oxide and 3'-hydroxycotinine in the rat (M ± SE).

Metabolite ratio	*Male*		*Female*	
	Noninduced	*Aroclor-induced*	*Noninduced*	*Aroclor-induced*
	(n = 5)	*(n = 5)*	*(n = 4)*	*(n = 5)*
CNN'O/TNN'O	9.4 ± 0.5	6.0 ± 0.5	7.2 ± 0.4	4.4 ± 0.5
THOC/CHOC	7.7 ± 0.4	13.6 ± 1.3	4.6 ± 0.3	9.0 ± 1.3

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Arch Toxicol (1988) 62: 395–397

Archives of
Toxicology
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Short Communication

Cotinine determination by immunoassays may be influenced by other nicotine metabolites

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Abstract. Polyclonal rabbit anticotinine antiserum, which can be used for biomonitoring nicotine uptake by the determination of cotinine in body fluids, was checked by a competitive ELISA for its cross-reactivity with nine nicotine metabolites. The highest percentage of relative cross-reactivity (about 30%) was observed with trans-3'-hydroxycotinine, a metabolite which is known to be excreted in 3-fold higher amounts than cotinine in the urine of human smokers. Therefore, it is possible that cotinine determinations performed by immunochemical methods – especially in urine – may yield overestimated cotinine concentrations.

Key words: Cotinine – 3'-Hydroxycotinine – Cross reaction – Enzyme-linked immunosorbent assay – Biomonitoring

Introduction

Several parameters have been proposed for biomonitoring the exposure of individuals to tobacco smoke. They have been reviewed recently by the International Agency for Research on Cancer (IARC monographs 1986), and with respect to environmental tobacco smoke by the National Research Council (NRC 1986) and Jarvis (1987). Among those parameters, the determination of nicotine uptake is the one most recommended, since nicotine is rather specific for tobacco smoke. Nicotine itself has a short biological half-life (about 1–2 h in human plasma; Benowitz et al. 1982; Kyerematen et al. 1982, Luck and Nau 1984). Since cotinine, a nicotine metabolite, has the advantage of a longer plasma half-life of 16–20 h (Benowitz et al. 1983), it has been widely used as a marker for nicotine uptake.

Sensitive methods for the determination of cotinine in biological fluids are available. Some of these are based on immunochemical techniques using specific antibodies. The antibodies used, however, should not cross-react with other compounds possibly present in the test fluid. Therefore, the mono- or polyclonal anticotinine antibodies have been tested for cross-reactivity with nicotine and nicotine metabolites (Langone et al. 1973; Langone and van Vunakis 1982; Bjerke et al. 1986, 1987; Hansel et al. 1986; van Vunakis et al. 1987).

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However, in none of these studies were the used anticotinine antisera tested for cross-reactivity with trans-3'-hydroxycotinine (THOC), a nicotine metabolite which is excreted in amounts 3 times higher than cotinine in the urine of human smokers (Neurath et al. 1987). The objective of this study was to investigate in a competitive cotinine ELISA the cross-reactivity of an anticotinine antiserum which was raised in rabbits according to the method described by Langone et al. (1973). Special emphasis was placed on cross-reactivity with the nicotine metabolite THOC.

Material and methods

Materials. The anticotinine antiserum was raised in rabbits by immunization with a thyroglobulin-cotinine conjugate. The conjugates of cotinine with thyroglobulin or human serum albumin (HSA) were prepared by reaction with trans-4'-carboxycotinine as described by Langone et al. (1973). Alkaline phosphatase-conjugated antirabbit IgG (goat) was purchased from Medac (Hamburg, FRG). *p*-Nitrophenylphosphate and the diethanolamine buffer were supplied by Boehringer (Mannheim, FRG), bovine serum albumin (BSA, fraction V) by Baker (Gross-Gerau, FRG), HSA by Sigma (Deisenhofen, FRG), and thyroglobulin and (−)-nicotine by Serva (Heidelberg, FRG). (−)-Cotinine was purchased from Roth (Karlsruhe, FRG). (−)-nicotine-1'-N-oxide, trans-4'-carboxycotinine, (±)-cis- and (±)-trans-3'-hydroxycotinine, (±)-demethylcotinine, and (−)-cotinine-1-N-oxide from Dr Georg Neurath (Hamburg, FRG). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

Buffers. PBS: phosphate buffered saline, 0.002 mol/l phosphate, 0.15 mol/l NaCl, pH 7.4; PBS-T: PBS supplemented with 0.05% (v/v) Tween 20; PBS-BSA-T: PBS-T supplemented with 0.1% (w/v) BSA.

Methods. The competitive ELISA for cotinine determination was performed in 96-well polystyrene microtiter plates (Immunoplate I, Nunc, Wiesbaden, FRG). The plates were coated with 50 µl cotinine-HSA conjugate diluted in 0.1 mol/l carbonate-bicarbonate buffer, pH 9.6, by incubation over night at 4° C. The wells were washed three times with PBS-T. Competitor solutions (100 µl) of various concentrations (concentration range see Table 1) were preincubated with 150 µl anticotinine antiserum

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Table 1. Relative cross-reactivity of anticotinine antibodies with nicotine metabolites

Compound	Concentration in assay (mol/l)			Steepness of inhibition curve[a] (absorbance units/log (mol/l))	Relative cross-reactivity (%)		
	Min. ($\times 10^{-9}$)	Max. ($\times 10^{-3}$)	At 50% inhibition ($\times 10^{-6}$)		This study[b]	Published data	
						Langone et al. (1982)[b]	Bjercke et al. (1986)[c]
(−)-cotinine	57	0.057	3.5	−0.40	100	100	100
1-N-methylnicotinium iodide	33	10	–	–	< 0.037	–	–
1'-N-methylnicotinium iodide	33	10	13 100	−0.58	0.027 ± 0.006[d]	–	–
nicotine-1'-N-oxide	56	17	9 660	−0.54	0.053 ± 0.006[d]	0.01	0.025
(−)-cotinine-1-N-oxide	52	16	1 880	−0.55	0.19 ± 0.01[d]	0.1	–
(−)-nicotine	120	10	1 360	−0.55	0.26 ± 0.04[d]	0.05	0.025
(+)-demethylcotinine	62	19	53.5	−0.39	6.6 ± 1.0[d]	0.5	6.9
(−)-cis-3'-hydroxycotinine	52	16	30.7	−0.48	11.5 ± 1.1[d]	–	–
(+)-trans-3'-hydroxycotinine	52	16	11.8	−0.45	29.6 ± 1.1[d]	–	–
(+)-nicotine	–	–	–	–	–	< 0.01	0.034
(+)-cotinine	–	–	–	–	–	< 0.01	–
(−)-nornicotine	–	–	–	–	–	0.01	0.20
(−)-cotinine-N'-oxide	–	–	–	–	–	–	2.8

Only published data regarding those metabolites which have an intact pyridine and pyrrolidine ring structure were selected

[a] At 50% inhibition

[b] Polyclonal antibodies raised in rabbits

[c] Monoclonal antibodies

[d] Mean ± standard deviation, the coefficients of variation range between 3.7% (for trans-3'-hydroxycotinine) and 22.2% (for 1'-N-methylnicotinium iodide)

(1:4000 diluted with PBS-BSA-T) for 30 min at room temperature (RT). After pipetting 50 µl of the preincubation mixtures into the wells of the coated microtiter plates, these were incubated for 1 h at RT and washed three times with PBS-T. Finally, 50 µl alkaline phosphatase conjugated antirabbit IgG (1:2000 diluted with PBS-BSA-T) was added to each well and the microtiter plate was incubated for 1 h at RT. After the plates were washed three times with PBS-T and one additional time with bidistilled water, 50 µl of freshly prepared substrate solution (10 mmol/l in diethanolamine buffer, pH 9.8, containing 0.5 mmol/l magnesium chloride) was added to each well. The enzyme reaction was performed for 30 min at RT in the dark and was stopped by adding 50 µl of 20% (w/v) NaOH solution. Absorbance was read at 405 nm.

For optimal comparison each individual nicotine metabolite was assayed together with cotinine on one microtiter plate. Duplicate or triplicate assays were performed on different days. The inhibition curves for cotinine and the other nicotine metabolites were calculated by a least square fit to the logistic function $y = A/(1+(B/x)^C)$ in which B represents the logarithm of competitor concentration at 50% inhibition. The logarithm of the competitor concentration and the value of the absorbance of the sample are given by x and y. A corresponds to the maximum absorbance (i.e. that at zero competitor concentration) and C determines the curvation of the concentration-response curve. The relative cross-reactivity was calculated from the molar competitor concentrations at 50% inhibition (Abraham 1969).

Results and Discussion

The nicotine metabolites demethylcotinine and cis- and trans-3'-hydroxycotinine showed moderate to marked cross-reactivity to the anticotinine antiserum (Table 1). It is of special interest that the highest cross-reactivity of about 30% was observed with THOC. High cross-reactivity would lead to an overestimation of cotinine in the presence of THOC and thus would explain the variations between the results which were obtained by determining the cotinine concentrations in urine by immunochemical versus gas chromatographic methods (Johnson et al. 1985; Biber et al. 1987; Letzel et al. 1987). Benowitz et al. (1983) has already discussed the possibility that the discrepancy between the cotinine half-life determined by gas chromatography ($t_{1/2}$ = 16–20 h) and that determined by radioimmunoassay ($t_{1/2}$ = 30–40 h, Langone et al. 1973) might be due to the different methods. In addition, they expected that "metabolites cross-reacting with cotinine antibodies might give a false long estimate of $t_{1/2}$ when measured by radioimmunoassay". The monoclonal anticotinine antibodies which have been produced and described (Bjercke et al. 1986) had not been tested for the cross-reactivity with THOC. Furthermore, these monoclonal antibodies do not seem to be more specific than the polyclonals raised in rabbits (Table 1). Additionally, anticotinine antibodies might have a high stereoselective specificity, as has been shown by Langone et al. (1982) for (+)- and (−)-cotinine (Table 1). Such a stereoselective specificity of anticotinine antibodies cannot be excluded for the cross-reactivity with (+)- and (−)-THOC. Since in the present study the cross-reactivity of THOC was assayed with its racemic form (±)-THOC, and since the naturally occurring metabolite (−)THOC (trans-1-methyl-3-(R)-hydroxy-5-(S)-3-pyridyl-2-pyrrolidinone, Dagne and Castagnoli 1972) is only part of the racemic (±)-THOC, the relative cross-reactivity of the anticotinine antiserum with the (−)-THOC enantiomer might be even higher than 30%.

Cross reactivity of anticotinine antibodies with further nicotine metabolites in the biological test fluids cannot be excluded, especially since, e.g. the whole spectrum of m...

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tine metabolites in the urine of smokers is not yet known (Neurath and Pein 1987). Therefore, one should be aware that biomonitoring the nicotine uptake by the determination of cotinine using immunochemical methods may produce questionable results concerning the cotinine concentrations.

Acknowledgements. The authors wish to thank Ms Gabriele Fleger and Heidi Bülles for helpful technical assistance.

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Received May 31, 1988/Received in revised form August 21, 1988/Accepted August 22, 1988

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- Appendix 7 -

Benzo[a]pyrene: Environmental Distribution and Human Exposure

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Benzo[a]pyrene: Environmental Distribution and Human Exposure

Incomplete combustion of organic matter represents the major source of polynuclear aromatic compounds (PAH) in the environment. PAHs are found at detectable concentrations in air, water, and soil samples of all types. Concentrations are typically small, in the order of μg/kg or ng/m^3. Since PAHs are highly lipophilic, they accumulate in organic fatty material and therefore have the potential to concentrate in the food chain.

Of the numerous PAHs, one compound that has perhaps received the most attention is benzo[a]pyrene (BaP). It is the focus of this discussion. The International Agency for Research on Cancer (IARC) has classified BaP as *probably carcinogenic to humans -- IARC Overall Evaluation 2A* (IARC, 1983, 1986a, 1986b). BaP has been identified in both mainstream and sidestream smoke from cigarettes, cigars, and pipes; marijuana smoke; and smoke-polluted environments (IARC, 1986b). Non-occupational inhalation exposure to BaP is primarily from tobacco smoke and urban air. However, Hattemer-Frey and Travis (1991) estimate that inhalation accounts for only 2% of the total daily intake of BaP. The focus of this paper therefore concerns the environmental distribution of BaP and human exposure to BaP.

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Atmosphere: Emission Sources of BaP

Osborne and Crosby (1987) cite the principal sources of BaP in the atmosphere as (1) coal- and oil-fired power stations, (2) domestic heating, (3) miscellaneous industrial processes, (4) vehicle exhausts, and (5) cigarette smoke, forest fires and volcanic activity. The yearly global emission of BaP is estimated to be about 5,000 tons, with the greatest contribution coming from coal combustion. BaP emissions in the U.S. have been estimated to be 1,260 tons/year, accounting for approximately 25% of the worldwide total (Grimmer, 1979).

As can be seen in **Table 1**, the major emission sources in the U.S. are heating and refuse burning. The percentages in **Table 1** are derived from a table presented by Grimmer (1979), reproduced herein as **Table 2**.

Since Osborne and Crosby (1987) cited cigarettes as a principal source of BaP emissions, an estimate was calculated of the tons emitted in sidestream smoke/year. This estimate is based on cigarette consumption/year in the U.S. (Tobacco Manufacturers Association, 1997), and uses the value of 147 ng/cig BaP in sidestream smoke (SS) (based on values for the 1R4F reference cigarette cited in R.J. Reynolds, 1988). **Figure 1** shows that for the years 1983-1996, the estimated emission of BaP in sidestream smoke to the atmosphere is less than 0.099 tons/year, which calculates to be less than 0.007% of the total estimated emissions in the U.S. Thus, SS is certainly not a major contributor to BaP in the atmosphere, compared to other sources.

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Table 1. Percentage by source of estimated BaP emission in the United States.

Emission source	Percentage
Automotive exhaust	1.7
Heating	38
Refuse burning	45
Industrial plants	16

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Table 2. Estimated B(a)P emissions in the United States, after Grimmer (1979).

Source	Tons/year	Total
Vehicle exhaust		
Gas-powered cars	10	
Gas-powered trucks	12	
Diesel fuel-powered trucks and buses	0.4	
		22.4
Heating		
Coal		
Hand-stoked residential furnaces	420	
Intermediate units	10	
Coal-fired steam power plants	1	
Oil		
Low-pressure air atomizer and others	2	
Gas	2	
Wood	40	
		475
Refuse burning		
Commercial, residential, institutional and apartments	33	
Open burning		
Forest and agricultural	140	
Vehicle disposal	50	
Coal refuse fires	340	
		563
Industrial plants		
Petroleum cracking	6	
Asphalt air-blowing	<1	
Coke production	192	
		200
Total (all sources)		1260

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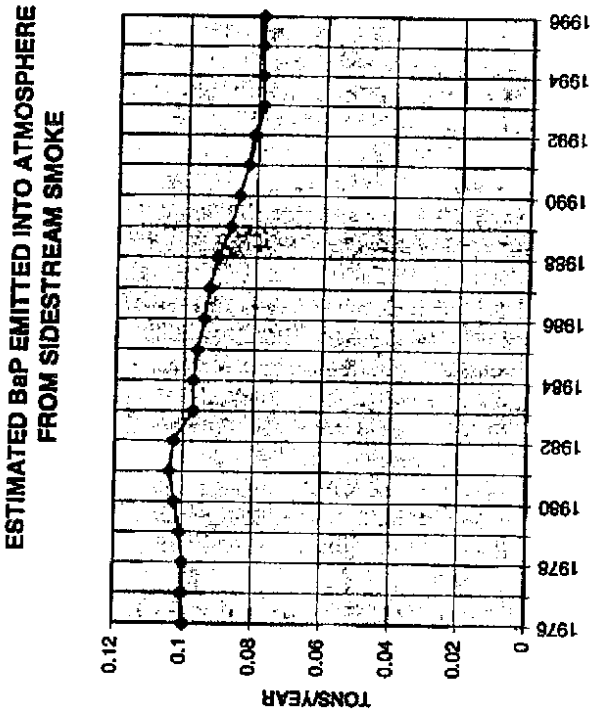

Figure 1.

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Occurrence of BaP in Air

The concentration of BaP in ambient air is dependent on a number of factors:

1) Season -- generally highest in winter and lowest in summer;

2) Source of emission -- industrial and transportation;

3) Meteorological factors;

4) Urban vs. rural settings; and

5) Geographic location (Europe vs. U.S.).

Table 3 and **Table 4** (after Pucknat, 1981) illustrates some of these factors; they are cited in the literature as being used for various calculations. The U.S. average for urban sites for the 5-year period 1966-1970 is about 2.0 ng/m^3 (Pucknat 1981, p. 85). The BaP concentration range in urban air of U.S. cities as determined by various authors in recent years (published during the period 1971-1977) is 0.13 to 3.2 ng/m^3 (Pucknat, 1981, p. 169). As can be seen in **Table 5**, BaP levels in European countries have historically been much higher than those reported in the U.S.; there is also a wide variation from winter to summer.

Pucknat (1981) cites a paper within a paper which reports a "safe" lifetime BaP dose for human lungs as 4.3 mg. On the basis of this value, he then states that the concentration of atmospheric BaP should not exceed 120 ng/m^3. A standard BaP concentration for industrial workers was determined to be 200 ng/m^3. (OSHA Workplace Exposure Limit (PEL) for coal tar pitch

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Table 3. **Average BaP concentrations (ng/m^3) in U.S. urban and rural areas (after Pucknat (1981), Table 5.14, p. 168).**

	1966	1970	1976
Urban	3.2	2.1	0.5
Rural	0.4	0.2	0.1

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Table 4. **Summer-winter average of ambient BaP concentrations (ng/m^3) in the air of selected cities (after Pucknat (1981), p. 169).**

City	BaP (ng/m^3)
Atlanta	4.5
Birmingham	15.7
Detroit	18.5
Los Angeles	2.9
Nashville	13.2
New Orleans	3.1
San Francisco	1.3

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Table 5. **Atmospheric benzo[a]pyrene concentrations (ng/m^3) for various locations around the world in summer and winter (after Osborne and Crosby (1987), Table 17.1, page 302).**

Location	Winter	Summer	Year
Sydney	8	0.8	1962-63
Liege, Belgium	110	15	1958-62
Ontario, Canada	15-20	1.2-18.5	1961-62
Prague	122	19	1964
Copenhagen	17	5	1956
Helsinki	5	22	1962-63
Paris	300-500		1958
Budapest	1000	32	1968
Teheran	6	0.6	1971
Belfast	51	9	1961-62
Milan	610	3	1958-60
Amsterdam	22	2	1968
	18	2	1969
	5		1970
	8		1971
Oslo	15	1	1956
Poland	130	30	1966-67
Madrid	120	0	1969-70
Stockholm	10	1	1960

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volatiles of 0.2 mg/m^3 averaged over an 8-hour workshift (Final Rule, January 1989); NIOSH recommended airborne exposure limit for coal tar pitch volatiles of 0.1 mg/m^3 over a 10-hour workshift; ACGIH recommendation that worker exposures, by all routes, be controlled to levels as low as can be reasonably achieved; New Jersey Hazardous Substance Fact Sheet -- Benzo(a)Pyrene -- Micromedex, Inc., 1974-1998.)

Water: Sources and Occurrences

According to a National Academy of Sciences (NAS) report (*Petroleum in the Marine Environment*, NAS 1975), about 6 million tons of petroleum hydrocarbons enter the oceans annually; the major contributors are marine transportation and runoff (urban and river). Other sources of PAHs in the oceans are coastal refineries, industrial and domestic waste, natural seeps, and atmospheric fallout. BaP levels found in water are shown in **Table 6** (after Osborne and Crosby, Table 17.5, p. 307).

As one can see, the levels vary significantly depending upon the sampling location and the type of water, but in general they are rather low. This is not unexpected since PAH compounds in solution are readily adsorbed on to the surface of dust, soil or other insoluble particles. These particles will fall slowly to the bottom, and thus PAH compounds are removed from solution. The levels of BaP reported in sediments on the other hand can be rather high in the order of μg/kg or even mg/kg of dry sample.

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Table 6. BaP levels in water.

Sample	Country	BaP (ng/L)
Tap water	FRG	0.25 - 9
Tap water	USA	0.2 - 1.6
Groundwater	FRG	1 - 10
Groundwater	USA	0.2
Rainwater	FRG	4 - 80
Reservoirs	UK	0.7 - 3.8
Well water	UK	0.2 - 0.6
Well water	FRG	2 - 15
Lake Erie	USA	0.3
River Rhine at Mainz	FRG	50 - 110
River Rhine at Koblenz	FRG	10 - 60
River Thames	UK	170 - 280
River Thames	UK	4.2 - 430
River Trent	UK	5.3 - 504
River Severn	UK	1.5 - 48
Ohio River	USA	5.6
Delaware River	USA	41.1
Motorway run-off	UK	570
Domestic effluent	FRG	38
Human urine		1300
Sewage sludge	FRG	1.7 (mg/kg)

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Soil: Sources and Occurrences

The majority of investigations of PAHs in soils have been carried out by Soviet investigators between 1967-1977; these papers only reported the BaP content (Osborne and Crosby, 1987). The concentrations of BaP measured in the U.S.S.R. ranged from 0.0008 mg/kg to 200 mg/kg, with the maximum value found in the vicinity of an oil refinery. Similarly high concentrations (650 mg/kg) were measured in the area of a carbon black factory. In samples of sandy and forest soil collected in West Germany, considerably lower concentrations of BaP, ranging from 0.001 to 0.0004 mg/kg, were found. The contamination of soil can be attributed almost exclusively to emissions from combustion processes. In the majority of surface soil samples taken in Iceland, where hardly any fossil fuels are burnt, the most commonly found PAHs were not detected (detection limit for BaP, e.g., 0.02 μg/kg soil). Soil samples taken at the Reykjavik, Iceland airport, however, were extremely contaminated, with BaP concentrations reaching 0.785 mg/kg.

"Human Exposure to Benzo(a)pyrene"

Hattemer-Frey and Travis (1991) used a multimedia transport model to evaluate environmental partitioning of BaP. Measured and predicted environmental concentrations were used to estimate the accumulation of BaP in the food chain and the subsequent extent of human exposure from inhalation and ingestion. Their results showed that the food chain is the dominant pathway of human exposure, accounting for about 97% of the total daily intake of BaP. See **Table 7**.

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Table 7. Pathways of human exposure to B(a)P (after Hattemer-Frey and Travis, 1991).

Source	Daily intake (μg/day)	% of total daily intake
Food (total)	2.1	97
Inhalation	0.05	2
Water	0.01	1
TOTAL	2.16	100

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This value of approximately 2.2 μg/day average daily intake of BaP is in agreement with other values reported in the literature (e.g., Suess, 1976). Hattemer-Frey and Travis (1991) then went on to discuss human exposure to BaP from smoking and indoor air pollution, referencing a paper by Butler and Crossley (1979) that reportedly estimated that one cigarette delivers approximately 39 ng of BaP. Further, Hattemer-Frey and Travis used in their calculations an estimate that the average smoker smokes 20 cigarettes per day. Based on these calculations, they suggested that the smoker receives an additional 780 ng/day (0.78 μg/day) BaP from smoking. Additionally, they again referenced Butler and Crossley (1979), who reported that concentrations of BaP measured indoors (2.2 ng/m^3) were comparable to outdoor air concentrations (2.5 ng/m^3); thus, indoor activities would not substantially increase the BaP intake, since inhalation is not a major pathway of human exposure to BaP.

Mainstream smoke concentrations of 9.2 ng BaP/cigarette have been reported for the Kentucky Reference Cigarette 1R4F (R.J. Reynolds, 1988). The value for a filtered cigarette could be rounded to 10 ng BaP/cigarette. Thus, an average (1 pack/day) smoker of filtered cigarettes would be exposed to an additional 0.2 μg/day of BaP, a level which is approximately 4 times the estimated daily intake of BaP by inhalation. **[See Table 8]** However, one should keep in mind that more than 90% of the daily intake of BaP is derived from other sources, primarily food.

This can be examined from the perspective of the daily lung burden as described by Chen and Thilly (1996). Instead of using the value of 10 m^3/day for breathing, the more widely used value of 20 m^3/day will be chosen. The value of 2.5 ng BaP/m^3 as the "urban air concentration" will

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Table 8. Estimated exposure of a smoker to BaP (ng/day).

	Cigarettes/day		
	10	**20**	**40**
1R4F filtered cigarette	100	200	400

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be used, which, as shown in **Table 9**, is probably an over-estimation of the urban air BaP concentration in the 1990s, if the downward trend has continued.

Using the formula (breathing rate/day X urban air BaP level) = ng BaP by inhalation/day, for a breathing rate of 20 m^3/day X 2.5 ng BaP/m^3 = 50 ng BaP/day or 0.05 μg/day by inhalation/day. The level of 0.05 ug/day by inhalation corresponds to what Hattemer-Frey and Travis reported **[See Table 7]**.

Examining BaP Exposure from ETS or Room-Aged Sidestream Smoke (RASS)

Attributing levels of PAH (BaP) in the air of restaurants, public rooms, etc., to ETS is difficult since other sources may be present, and other factors, such as ventilation rates, number of smokers, etc., may confound the issue. However, Grimmer (1983), under controlled conditions, reported 22 ng/m^3 BaP where cigarettes were being smoked and less than 3 ng/m^3 where no cigarettes were being smoked. He calculated that ETS contributed about 7 times the background BaP level. Grimmer states that "the measured concentrations of 22 ng BaP per cubic meter has to be considered as a maximum BaP concentration attainable by smoking. In practice nobody would tolerate this concentration" of smoke due to eye irritation, etc.

A 12-month inhalation study in rats using room-aged sidestream smoke (RASS) (INBIFO, data enclosed) reports the following concentrations of BaP in RASS from 1R4F cigarettes: 0.13 μg/m^3 (upper limit) for the whole-body 12-month exposure group, and 0.121 μg/m^3 (upper

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Table 9. **Average BaP concentrations (ng/m^3) in U.S. urban and rural areas during 1966-1977 (after Pucknat 1981, Table 5.14, p. 168).**

Location	Year		
	1966	1970	1976
Urban	3.2	2.1	0.5
Rural	0.4	0.2	0.1

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limit) for the head-only 12-month exposure group (Haussmann et al., 1998). Again, if we consider urban ambient air to contain an average of 2.5 ng/m^3 BaP, one can see that the rats in this study were exposed to approximately 50 times the level of BaP found in ambient air. The RASS concentrations in this study were approximately 100-fold higher than the maximum of the average concentrations of respiratory suspended particles (RSP) reportedly attributable to ETS (Guerin et al., 1992; U.S. EPA. 1992; Jenkins et al., 1996). Thus, if one assumes that BaP, which is in the particulate phase, tracks with RSP, then 0.13 μg/m^3 would correspond to 0.0013 μg/m^3. From the perspective of human exposure, this level of exposure would be equivalent to 0.0013 μg/m^3 X 20 m^3/day = 0.026 μg/ or 26 ng/day, which is about half the daily level by inhalation estimated by Hattemer-Frey and Travis.

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- Appendix 8 -

Section on Carcinogenesis from
Philip Morris U.S.A. Comments on
Health Effects of Exposure to Environmental Tobacco Smoke
Final Draft for Scientific, Public and SRP Review
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency
May 5, 1997

March 20, 1998

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COMMENTS ON:

Health Effects of Exposure to Environmental Tobacco Smoke

Final Draft for Scientific, Public and SRP Review

Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

PHILIP MORRIS U.S.A.

May 5, 1997

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COMMENTS ON CHAPTER 7 -- CARCINOGENIC EFFECTS

Summary Overview

Based on Chapter 7, "Carcinogenic Effects," OEHHA contends that (1) the data support a causal relationship between ETS exposure and lung cancer, (2) the data support a causal relationship between ETS exposure and cancer of the nasal sinus and (3) the data are "suggestive" of a causal relationship between ETS exposure and cervical cancer. Philip Morris herein provides comments on those three conclusions. Given the limited time made available for public comments, Philip Morris has not been able to address in detail the other cancer endpoints discussed by OEHHA; this does not mean that the Company endorses OEHHA's conclusions or the reasoning described therein, or that Philip Morris believes that OEHHA's review is complete, impartial, or appropriate. For instance, Philip Morris notes that OEHHA has apparently not considered a number of potentially relevant references that could contribute to its discussion of non-lung cancers; thus, the review in Chapter 7 is incomplete on this basis alone. A list of those references is provided as **Attachment A** to this comment.

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Section 7.2 -- ETS and Lung Cancer

Appendix A of the Final Draft purports to be a "Summary of Public Comments and Responses" received on previously released draft chapters of the document. Pages A-2 and A-3 are described as a "List of Those Commenting"; however, this list is incomplete. In particular, the Comment of Philip Morris U.S.A. on the January 1996 External Review Draft, *Carcinogenic Effects of Exposure to Environmental Tobacco Smoke -- Excerpt: ETS and Lung Cancer*, is not included on the list. In so omitting the Comment of Philip Morris, OEHHA has failed to respond to the many issues raised therein, as it is required to do. Moreover, the essence of the discussion on lung cancer in the Final Draft is unchanged from the January 1996 External Review Draft. Therefore, the Comment already submitted by Philip Morris on the lung cancer claims made by OEHHA is still relevant. That Comment is resubmitted as **Attachment B** to this submission.

In the Comment on lung cancer already submitted by Philip Morris, a number of articles overlooked by OEHHA in its previous draft were referenced; however, OEHHA has failed to incorporate most of those references into the Final Draft document. **Attachment C** to this Section of this Comment lists those articles. It is incumbent upon OEHHA to address the important issues raised by these articles.

In the year since Philip Morris submitted the Comment that OEHHA ignored, additional relevant literature has accumulated in the area of ETS exposure and lung cancer. **Attachment D** to this Comment lists new lung cancer literature, which raises a number of important

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issues relevant to OEHHA's claims. OEHHA should review this literature and incorporate it into the ETS assessment.

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Section 7.3.1 -- Nasal Sinus Cancer

OEHHA Claim: Nasal sinus cancer is listed in Table ES.1 (p. ES-2) under "effects ***causally*** associated with ETS exposure." [emphasis added]

Response:

- Nowhere does OEHHA indicate how it reached a conclusion of "causality." In fact, OEHHA's statements in the text of the document do not even mention a causal relationship.

- The reader of the OEHHA document is led to assume that the conclusion of "causality" is scientifically justified -- because it appears in the Executive Summary of the document -- when, in actuality, OEHHA provides ***no*** rationale at all for such a conclusion, and when the Executive Summary states a conclusion different from the conclusions stated in the text of the document.

- In the text, OEHHA states: "Of the studies examining the effect of ETS exposure on nasal sinus cancers, all three show consistent associations, presenting strong evidence that ETS exposure increases the risk of nasal sinus cancers in nonsmoking adults" (p. ES-7) and "Existing studies consistently show a significant positive association between exposure to ETS and nasal sinus cancer in nonsmokers. The results have been observed in studies conducted in eastern and western countries, in males and females, in cohort and case-control study designs, and with some adjustment for possible confounders." (p. 7-28)

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- OEHHA should formally indicate how it reached the determination of causality that warranted the inclusion of nasal sinus cancer in Table ES.1.

OEHHA Claim: "Active smoking is firmly established as a causal factor for cancers of the . . . nasal sinus cavity." (p. 7-26)

Response:

- No references are provided for this contention. However, the 1982 and 1989 Surgeon General's reports and the 1986 IARC Monograph (No. 38), cited elsewhere in the chapter by OEHHA, do not even mention nasal sinus cancer in their discussions of cancers purportedly associated with active smoking.

OEHHA Claim: "There are some data on the role of ETS for other cancer sites. including cancers of nasal sinus cavity . . ." (p. 7-26)

- The 1982 and 1989 Surgeon General's Reports, cited by OEHHA, do not even mention nasal sinus cancer in their discussions of ETS.

OEHHA Claim: "Existing studies consistently show a significant positive association between exposure to ETS and nasal sinus cancer in nonsmokers." (p. 7-28)

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Response:

- ***Lack of Statistical Significance:*** Only one of the risk estimates cited by OEHHA in its discussion of the studies has a confidence interval that excludes 1.0. The single risk estimate that is "statistically significant" has an extremely wide confidence interval, based as it is on only nine cases.

- In Table 7.8 (p. 7-65), OEHHA lists the reported risk estimates from the three nasal sinus cancer studies. The table clearly shows that ***only one of the reported risk estimates has a confidence interval that excludes 1.0.***

- None of the three spousal smoking risk estimates cited by OEHHA from the Hirayama study is statistically significant -- all the confidence intervals ***include*** 1.0. Thus, those risk estimates are consistent with neither an increase or a decrease in risk.

- Fukuda and Shibata do not even discuss the statistical significance of the individual risk estimates in their paper, but OEHHA has calculated confidence intervals. The great width (1.7 to 19.4) of the CI on the risk estimate for more than one smoker in the home illustrates the uncertainty associated with that risk estimate. When coupled with the fact that it is based on only nine cases, this makes any interpretation based on that number highly suspect.

- The authors of the Zheng, et al., study provide only limited information on the spousal smoking analysis. Moreover, the confidence intervals for both risk estimates presented

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overlap 1.0 and are quite wide (as the authors themselves acknowledge), particularly the one for maxillary sinus cancers.

- ***Sample Size:*** OEHHA's conclusion of causality is based on three studies reporting data based on only 91 cases in total. Neither the number of studies nor the number of cases is sufficient support for such a conclusion.

- OEHHA even notes that the Hirayama (1983) study results are based on only 28 cases, but fails to point out that such a small sample size compromises the interpretation of the reported results. The low number of subjects results in very unstable risk estimates.

- Fukuda and Shibata (1990) acknowledge that their data set was of "relatively small size." However, OEHHA's discussion of this study obscures the fact that there are only 35 nonsmoking cases. OEHHA focuses on the risk estimates by exposure category and the purportedly statistically significant "trend," while failing to acknowledge the fragility of those risk estimates, based as they are on 15 and nine cases, respectively.

- While the Zheng, et al., study is fairly large (particularly given the rarity of this tumor), the risk estimates reported for spousal smoking are based on only 28 cases (those who were nonsmokers). Again, sample size is a distinct limitation of this study, a point which OEHHA fails to clearly state in their summary of the paper.

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OEHHA Claim: "The results have been observed in studies . . . with some adjustment for potential confounders." (p. 7-28)

Response:

- OEHHA states that the risk estimates from the Hirayama study included an adjustment for ***husband's*** age. Hirayama has adopted this approach consistently in his analyses of data from this study, when, as it has been noted elsewhere, "[i]t is absolutely routine in epidemiology to standardize for the age of the ***subject***." [emphasis added] (Lee, 1992) Hirayama's odd approach would seem to raise questions about the overall validity of his study.

- Cancer of the paranasal sinuses is more common in Japan than in the United States. (DeVita, et al., 1993; for references to materials not cited by OEHHA, see **Attachment A)** This would imply that caution is warranted in applying the reported results of the Hirayama study (as well as the Fukuda and Shibata study) to the United States population. There may well be ethnic-specific and lifestyle risk factors that have not been considered, as have been reported for nasopharyngeal cancer. (Schottenfeld and Fraumeni, 1996)

- While Hirayama writes in the paper cited by OEHHA that "[n]o other risk factors studied significantly altered the risk of nasal sinus cancer in women," he fails to provide the reader with information as to what those risk factors might be. (Hirayama, 1983) Possible risk factors for this cancer have been called "multifold," and reportedly include exposure to

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nickel; occupation in the furniture, textile, and shoe industries; occupational exposures to chromium, mustard gas, isopropyl alcohol, and radium; and possibly chronic sinusitis. (DeVita, et al., 1993)

OEHHA Claim: "The results have been observed in studies conducted in eastern and western countries, in males and females . . ." (p. 7-28)

Response:

- OEHHA's statement misrepresents the available data, reported for only one sex in each of two countries.

- Two studies present data for nonsmoking ***Japanese women*** (Hirayama; Fukuda and Shibata) and one for nonsmoking ***white American men*** (Zheng, et al.). The Japanese studies do not have data on nonsmoking men, and the U.S. study does not have data on women.

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Section 7.4.1. -- Cervical Cancer

OEHHA Claim: Cervical cancer is listed under "effects with suggestive evidence of a causal association with ETS exposure" in Table ES.1. (p. ES-2)

Response:

- Nowhere does OEHHA indicate how it reached a conclusion of "suggestive" evidence for "causality." In fact, OEHHA's statements in the text of the document do not even mention a causal relationship.

- The Table ES.1 conclusion is not justified or supported by the analysis presented in Chapter 7, which only states: "There is supportive evidence from epidemiological and biochemical studies implicating a role for ETS exposure in the etiology of cervical cancer in nonsmokers." (p. 7-32) OEHHA presents no criteria for extrapolating from this position to a conclusion that there is "suggestive" evidence for "causality," nor does it define what exactly constitutes "supportive" evidence or "suggestive" evidence.

- The reader of the OEHHA document is led to assume that the conclusion of "suggestive" evidence for "causality" is scientifically justified -- because it appears in the Executive Summary of the document -- when, in actuality, OEHHA provides ***no*** rationale at all for such a conclusion, and when the Executive Summary states a conclusion different from the conclusions stated in the text of Chapter 7.

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- OEHHA should formally indicate how it reached the determination of "suggestive" evidence for causality that warranted the inclusion of cervical cancer in Table ES.1.

- OEHHA states in Chapter 7: "[H]uman papillomarivus [sic] . . . has been accepted as the sexually transmitted etiological factor in cervical cancer." (p. 7-28) OEHHA thereby seemingly acknowledges the scientific consensus on the importance of the sexually-transmitted agent, human papillomavirus (HPV), in the etiology of cervical cancer. (See **Attachment A**: Winklestein, 1990; Bosch, et al., 1995; Munoz, et al., 1996; Bosch, et al., 1996) In fact, a consensus panel of the National Institutes of Health has concluded that cervical cancer is "causally related" to infection with HPV. (McNeil, 1996)

- Given the general acknowledgment of HPV as a cause of cervical cancer, it is curious that OEHHA would even evaluate ETS exposure in terms of "causality," as indicated in Table ES.1.

- Moreover, a noted cancer textbook refers to active smoking as a "cofactor" for cervical cancer (DeVita, et al., 1993); given this position, it clearly seems very premature to conclude that there is suggestive evidence that ETS is ***causally*** associated with this disease, as OEHHA claims.

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OEHHA Claim: "The relationship between ETS exposure and cervical cancer was investigated in one cohort and three case-control studies." (p. 7-29)

Response:

- OEHHA's review of the literature on ETS exposure and cervical cancer is incomplete. When additional studies are taken into account, however, no clear picture of "risk" emerges. What seems most likely, based upon the data from these and other studies, is that ETS exposure is a marker for a number of interrelated factors, and that the reportedly elevated risk estimates represent the residual effect of insufficient control for these variables.

- Munoz, et al. (1996), report cervical cancer risk estimates for husband's smoking status that are not statistically significant when adjusted for a number of potential confounding factors. OEHHA did not reference this article.

- Bosch, et al. (1996), report statistically significant cervical cancer risk estimates for husband's smoking, but conclude that the "two most likely reasons" for their reported results are misclassification of human papillomavirus exposure and insufficient adjustment. OEHHA did not reference this article.

OEHHA Claim: "Positive associations were observed in two of three case-control studies and a statistically nonsignificant positive association was observed in the only cohort study conducted." (p. ES-7)

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Response:

- ***Misleading Presentation of Data:*** Table 7.9 (p. 7-66) is incomplete, reporting data from only two of the available studies (Slattery, et al.; Coker, et al.). Nevertheless, Table 7.9 (p.7-66) clearly shows that ***only one cited risk estimate from the two studies is statistically significant.*** Chance has thus not been excluded as an explanation for the overwhelming majority of the reportedly increased risks cited by OEHHA in that table.

- The series of risk estimates OEHHA chose to include in Table 7.9 (p. 7-66) includes the largest risk estimate reported by Slattery, et al., for nonsmokers. The only statistically significant risk estimates for ETS exposure reported in Table 6 of the Slattery, et al., paper are for the highest exposure group for "all" reported ETS exposure or for home ETS exposure. This pattern would be consistent with confounding factors being clustered in that exposure group.

- ***Sample Size:*** It is also clear from Table 7.9 (p. 7-66) that the risk estimates for different ETS "exposure levels" are highly suspect, based as they are on very few cases.

- Moreover, the Slattery, et al., study, which seems to be key to OEHHA's contentions, fails to even provide the numbers of cases and controls for the different levels. This incomplete reporting makes interpretation of the study's reported results quite difficult.

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OEHHA Claim: "There is supportive evidence from epidemiological . . . studies implicating a role for ETS exposure in the etiology of cervical cancer in nonsmokers." (p. 7-32)

Response:

- Many questions remain about the ETS-cervical cancer epidemiologic studies cited by OEHHA, making any summary interpretation of their reported conclusions difficult. Many of the reported risk estimates are not statistically significant. The studies have generally (in some cases, completely) failed to adequately consider potential confounding factors. Given these limitations, OEHHA's conclusion is unwarranted.

OEHHA Claim: "The current chapter extends or modifies the discussion of issues related to ETS exposure and cervical cancer (*e.g.*, on confounders in epidemiological studies . . .)" (p. iii)

Response:

- Compared to the cervical cancer section in the May 1994 External Review Draft, the current Chapter 7 does include some "modified" discussion of confounders, in that they are now briefly mentioned in the context of the data collected in the epidemiologic studies cited by OEHHA.

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- However, OEHHA's treatment of confounders can certainly be further "extended." This is perhaps the most important issue in evaluating the cervical cancer studies. and OEHHA pays completely insufficient attention to it.

- ***Inadequate Treatment of Confounders in Individual Studies:*** The discussion in Chapter 7, plus statements from other reviews, clearly indicates shortcomings in the available literature's treatment of confounding factors.

- It has been stated about the Hirayama study:

> [T]here are a number of doubts about the results from this large prospective study, the most important of which appear to be incompleteness of follow-up and doubts about the validity of the statistical methods used. ***Failure to gather data on confounding variables relevant to specific cancers is also a problem.*** [emphasis added] (Lee, 1992)

OEHHA even acknowledges that Hirayama's ETS data "were not adjusted for potential confounders including subjects' or husband's sexual activity" (p. 7-29). Given what is now known about the relationship between HPV and cervical cancer, this failure on Hirayama's part means that this study can be treated as providing essentially no information relevant to the question of ETS exposure and cervical cancer risk.

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- With respect to the Sandler, et al., study, Lee (1992) notes:

> Apart from . . . the problems of multiple testing, it should be realized that the Sandler I study collected ***no data*** on sexual habits, which are strongly correlated both with risk of cervix cancer and with smoking habits. [emphasis added]

OEHHA acknowledges this as well: "[I]nformation typically obtained in studies of a specific cancer site (*e.g.*, sexual activity in studies of cervical cancer) ***was not collected.***" [emphasis added] (p. 7-29) As with the Hirayama study, the Sandler study can be given little weight in analyses of cervical cancer claims.

- Studies report that both personal smoking behavior and ETS exposure are correlated with a number of other lifestyle factors (related to sexual activity and alcohol and drug use) that appear to be markers for an increased risk of HPV infection. Disentangling the possible effects of these potential confounders is difficult, and the epidemiologic studies to date have not adequately addressed them.

- In a letter to the editor on the Slattery, et al., study, Zang, et al., note:

> In this report, there clearly is undermatching of control patients with regard to important risk factors including sexual activity, religious background, and education. . . . Since the previously mentioned risk factors are correlated highly with one another as well as with active and passive smoking, the risk estimates relating smoking and cervical cancer may be subject to substantial bias and confounding. . . . The effect of adjustment on odds ratios is far greater than expected in case-control studies of this sort, as, for example, the decrease from 14.84 to 2.96 for passive smoke exposure. ***In fact, the adjusted odds***

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> ***ratios are probably no more than the leftover effect of variables controlled imperfectly by logistic regression.*** [emphasis added]

- Zang, et al., also note:

> The apparent association between cervical cancer and environmental smoke exposure may, in fact, be the result of increased exposure to papillomaviruses or herpes simplex virus 2 through contact with male sexual partners who smoke, since one might expect smokers to be generally more sexually active than nonsmokers, and case patients are more sexually active than control subjects.

- The author of a 1992 book on ETS chronic disease claims stated about the Slattery, et al., study:

> [T]he major problem with interpreting this finding as cause and effect is the extreme difficulty of adequately adjusting for sexual habits. . . . Since the number of sexual partners is only an inaccurately measured surrogate of the true sexually related cause of cervix cancer . . . the adjustment will be incomplete and leave a residual confounding effect. ***It is entirely plausible that the whole of the adjusted relative risk could be explained by this.*** [emphasis added] (Lee, 1992)

- That potentially misleading results can come from studies with inadequate attention to confounding factors (e.g., Hirayama, as acknowledged by OEHHA; Slattery, as pointed out by other reviewers) seems clear when the Coker, et al. (1992), study is compared to those other studies. In that study, the authors included age, race, education, number of Pap smears, number of sexual partners, and history of genital warts as confounders in their analysis of ETS exposure and cervical cancer risk. As for their reported results, they note, "the

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confidence intervals associated with each of these adjusted odds ratios were ***wide and clearly not statistically significant.***" [emphasis added]

- ***Omission of Relevant Data:*** In the discussion of cervical cancer, OEHHA completely overlooked two articles presenting relevant data on the potential for confounding in the epidemiologic studies. Given OEHHA's predilection for data from California elsewhere in the report, this omission seems particularly odd.

- Holly, et al. (1992), report data which "confirm that cigarette smoking is strongly associated with numerous life-style and behavioral factors, many of which have been linked directly or indirectly with cervical cancer." These data were collected from women residing in the San Francisco Bay area.

- In another study from the same research group, Cress, et al. (1994), investigated whether women exposed to ETS differed from non-exposed women on a number of characteristics. This study was based on self-report of ETS exposure, just as are the epidemiologic studies of cervical cancer risk. Several statistically significant differences between "ETS-exposed" and non-exposed women were reported; they included marijuana use, beer consumption, and being divorced or separated. The authors concluded:

> [W]omen nonsmokers exposed to passive smoke were different from those not exposed. Studies that examine the association between passive smoking and disease need to measure dietary, lifestyle,

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sexual, and reproductive factors to adequately allow for these differences.

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Attachment A to Comments on Chapter 7 -- Carcinogenic Effects

Studies on Cancers Other than Lung Not Considered by OEHHA

1. Birch, J.M., et al., "The Inter-Regional Epidemiological Study of Childhood Cancer (IRESCC): Case-Control Study of Children with Central-Nervous-System Tumours," *Brit J Neurosurg* 4: 17-26, 1990.

2. Bosch, F.X., et al., "Prevalence of Human Papillomavirus in Cervical Cancer: A Worldwide Perspective," *J Natl Cancer Inst* 87: 796-802, 1995.

3. Bosch, F.X., et al., "Male Sexual Behavior and Human Papillomavirus DNA: Key Risk Factors for Cervical Cancer in Spain," *J Natl Cancer Inst* 88: 1060-1067, 1996.

4. Bunin, G.R., et al., "Risk Factors for Astrocytic Glioma and Primitive Neuroectodermal Tumor of the Brain in Young Children: A Report from the Children's Cancer Group," *Cancer Epidemiol Biomarkers & Prev* 3: 197-204, 1994.

5. Cordier, S., et al., "Incidence and Risk Factors for Childhood Brain Tumors in the Ile de France," *Int J Cancer* 59: 776-782, 1994.

6. Cress, R.D., "Characteristics of Women Nonsmokers Exposed to Passive Smoke," *Prev Med* 23: 40-47, 1994.

7. DeVita, V.T., et al., (eds.)., *Cancer: Principles & Practice of Oncology*, 4th Edition, Philadelphia, J.B. Lippincott Company, 1993.

8. Filippini, G., et al., "Mothers' Active and Passive Smoking During Pregnancy and Risk of Brain Tumors in Children," *Int J Cancer* 57: 769-774, 1994.

9. Grufferman, S., et al., "Parents' Use of Cocaine and Marijuana and Increased Risk of Rhabdomyosarcoma in Their Children," *Cancer Causes and Control* 4: 217-224, 1993.

10. Gurney, J.G., et al., "Childhood Brain Tumor Occurrence in Relation to Residential Power Line Configurations, Electric Heating Sources, and Electric Appliance Use," *Am J Epidemiol* 143: 120-128, 1996.

11. Hellberg, D., et al., "Smoking and Cervical Intraepithelial Dysplasia: An Association Independent of Sexual and Other Risk Factors," *Acta Obstet Gynecol Scand* 65: 625-631, 1986.

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12. Hirayama, T., "Passive Smoking and Cancer: The Association Between Husbands Smoking and Cancer in the Lung of Non-smoking Wives," in: *Indoor Air Quality*, ed., H. Kasuga, Berlin, Heidelberg, Springer-Verlag, 299-311, 1990.

13. Hirose, K., et al., "Subsite (Cervix/Endometrium)-Specific Risk and Protective Factors in Uterus Cancer," *Japan J Cancer Res* 87: 1001-1009, 1996.

14. Holly, E.A., et al., "Characteristics of Women by Smoking Status in the San Francisco Bay Area," *Cancer Epidemiol Biomarkers & Prev* 1: 491-497, 1992.

15. Hurley, S.F., et al., "Tobacco Smoking and Alcohol Consumption as Risk Factors for Glioma: A Case-Control Study in Melbourne, Australia," *J Epidemiol Comm Health* 50: 442-446, 1996.

16. Ji, B.T., et al., "Paternal Cigarette Smoking and the Risk of Childhood Cancer Among the Offspring of Nonsmoking Mothers," *J Natl Cancer Inst* 89: 238-244, 1997.

17. Kilpatrick, S.J., "Re: Prenatal Exposure to Parents' Smoking and Childhood Cancer," *Am J Epidemiol* 135: 712, 1992.

18. Kreiger, N., et al., "Risk Factors for Renal Cell Carcinoma: Results of a Population-Based Case-Control Study," *Cancer Causes and Control* 4: 101-110, 1993.

19. Layard, M., "Environmental Tobacco Smoke and Cancer: The Epidemiologic Evidence," in: *Environmental Tobacco Smoke: Proceedings of the International Symposium at McGill University 1989*, eds., D. Ecobichon and J. Wu, Lexington, Mass., Lexington Books, 99-115, 1990.

20. Layde, P., "Smoking and Cervical Cancer: Cause or Coincidence?" *JAMA* 261: 1631-1633, 1989.

21. Lee, P., "Other Cancers," in: *Environmental Tobacco Smoke and Mortality*, Basel, Karger, 168-183, 1992.

22. Lee, P.N., "Re: Prenatal Exposure to Parents' Smoking and Childhood Cancer," *Am J Epidemiol* 135: 713-714, 1992.

23. Linet, M.S., Gridley, G., Cnattingius, S., Nicholson, H.S., Martinsson, U., Glimelius, B., Adami, H.O., and Zack, M., "Maternal and Perinatal Risk Factors for Childhood Brain Tumors (Sweden)," *Cancer Causes and Control* 7: 437-448, 1996.

24. Liu, L., et al., "Early Life Experience and Other Risk Factors for Breast Cancer in Chinese Never-Smoking Women," *Epidemiology* 7(4, Suppl.): S37, 1996.

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25. London, S.J., et al., "Exposure to Residential Electric and Magnetic Fields and Risk of Childhood Leukemia," *Am J Epidemiol* 134: 923-937, 1991.

26. McCredie, M., et al., "Perinatal and Early Postnatal Risk Factors for Malignant Brain Tumours in New South Wales Children," *Int J Cancer* 56: 11-15, 1994.

27. McNeil, C., "Consensus Panel on Cervical Cancer Highlights the HPV Connection," *J Natl Cancer Inst* 88: 575, May 1, 1996.

28. Munoz, N., et al., "Difficulty in Elucidating the Male Role in Cervical Cancer in Colombia, a High-Risk Area for the Disease," *J Natl Cancer Inst* 88: 1068-1075, 1996.

29. National Cancer Institute, Office of Cancer Communications, "Abortion and Possible Risk for Breast Cancer: Analysis and Inconsistencies," Press Release, October 26, 1994.

30. Norman, M.A., et al., "Prenatal Exposure to Tobacco Smoke and Childhood Brain Tumors: Results from the United States West Coast Childhood Brain Tumor Study," *Cancer Epidemiol Biomarkers & Prev* 5: 127-133, 1996.

31. Preston-Martin, S., et al., "Maternal Consumption of Cured Meats and Vitamins in Relation to Pediatric Brain Tumors," *Cancer Epidemiol Biomarkers & Prev* 5: 599-605, 1996.

32. Ross, J.A., "Epidemiology of Childhood Leukemia, with a Focus on Infants," *Epidemiol Reviews* 16: 243-272, 1994.

33. Schottenfeld, D., and Fraumeni, J.F., *Cancer Epidemiology and Prevention*, Second Edition, New York, Oxford University Press, 1997.

34. Schymura, M.J., et al., "A Case-Control Study of Childhood Brain Tumors and Maternal Lifestyle," *Am J Epidemiol* 143(11, Suppl.): S8, 1996.

35. Shu, X.O., et al., "Parental Alcohol Consumption, Cigarette Smoking, and Risk of Infant Leukemia: A Childrens [sic] Cancer Group Study," *J Natl Cancer Inst* 88: 24-31, 1996.

36. Shu, X.O., "A Population-Based Case-Control Study of Childhood Leukemia in Shanghai," *Cancer* 62: 635-644, 1988.

37. Sorahan, T., et al., "Childhood Cancer and Parental Use of Alcohol and Tobacco," *Ann Epidemiol* 5: 354-359, 1995.

38. Sorahan, T., et al., "Childhood Cancer and Parental Use of Tobacco: Deaths from 1953 to 1955," *Brit J Cancer* 75: 134-138, 1997.

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39. Tredaniel, J., et al., "Environmental Tobacco Smoke and the Risk of Cancer in Adults," *Eur J Cancer* 29A(14): 2058-2068, 1993.

40. Tredaniel, J., et al., "Exposure to Passive Smoking During Pregnancy and Childhood, and Cancer Risk: The Epidemiological Evidence," *Paediatr Perinat Epidemiol* 8: 233-236, 1994.

41. Vaughan, T.L., et al., "Nasopharyngeal Cancre in a Low-Risk Population: Defining Risk Factors by Histological Type," *Cancer Epidemiol Biomarkers Prev* 5: 587-593, 1996.

42. Wells, A., "Re: 'Breast Cancer and Cigarette Smoking: A Hypothesis,'" *Am J Epidemiol* 135(10): 1184-1186, 1991.

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Attachment B to Comments on Chapter 7 – Carcinogenic Effects

Philip Morris Comment on 1996 External Review Draft

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PHILIP MORRIS U.S.A.

COMMENTS ON:

CARCINOGENIC EFFECTS OF EXPOSURE TO ENVIRONMENTAL TOBACCO SMOKE

EXCERPT: ETS AND LUNG CANCER

California Environmental Protection Agency
Reproductive and Cancer Hazard Assessment Section
Office of Environmental Health Hazard Assessment

External Review Draft
January 1996

April 1, 1996

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SECTION I: OVERVIEW AND INTRODUCTION

In the January 1996 external review draft, "Carcinogenic Effects of Exposure to Environmental Tobacco Smoke -- Excerpt: ETS and Lung Cancer" (hereinafter, the Excerpt), Cal/EPA contends that recently published epidemiologic studies support the conclusions reached in the U.S. EPA's 1993 Risk Assessment on environmental tobacco smoke (ETS). This contention is incorrect and is not supported by the data.

In this written comment, it will be well documented that the present draft of the Excerpt is a superficial treatment of the claimed association between ETS exposure and lung cancer risk. (Section II) Cal/EPA selectively cites and interprets studies and data in an attempt to support a conclusion that the recent epidemiologic data agree with the positions espoused in the U.S. EPA Risk Assessment on ETS.

Further, this comment will highlight and summarize a number of the unresolved criticisms of the U.S. EPA Risk Assessment on ETS, showing that the assumption that that document was a conclusive, thorough review of the literature is unfounded. (Section III) In particular, data on ETS chemistry, ETS exposure data, the limitations of questionnaire data, animal inhalation studies and genotoxicity data were not adequately addressed.

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Finally, since Cal/EPA relies almost exclusively on the epidemiologic literature in reaching its conclusions, this comment will address a number of epidemiologic issues that were treated in insufficient detail in the Excerpt. (Section IV)

Given the shortcomings of the Excerpt, this comment concludes that Cal/EPA's stated position is not adequately supported. Specifically, when all of the relevant data are considered, those data **do not support the claim that ETS exposure is associated with an increased risk of lung cancer in nonsmokers.**

Based on the information contained herein and upon publicly available information, Cal/EPA should defer from taking any further action on this assessment of risks.

- To avoid "bad science," the Cal/EPA report should await the recommendations of the OEHHA Risk Assessment Advisory Committee. Cal/EPA Secretary James Strock has been quoted as saying that "scientifically sound risk assessments are the foundation upon which wise environmental decisions are made," which implies an obligation to assure that this report is sound science.

  (i) The California Legislature has also recognized the significant impact of risk assessments. In 1993, the

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Legislature enacted S.B. 1082 (Cal. Health & Safety Code sec. 57004), which required the director of Cal/EPA's Office of Environmental Health Hazard Assessment (OEHHA) to convene an advisory committee "to conduct a comprehensive review of the policies, methods, and guidelines to be followed by the state for the identification and assessment of chemical toxicity." The Advisory Committee was convened in 1994. It has held seven meetings; the next meeting will be held on April 10 and 11, 1996. At the same time that the work of the Advisory Committee is nearing completion, Cal/EPA is attempting to produce a formal risk assessment concerning ETS, complete with conclusions that may be calculated to command attention in the press but may be easily misinterpreted as to their true significance.

Given the universally recognized need for thorough and reasonable risk assessment guidelines,[1] it is difficult to see how Cal/EPA would now allow the release of any risk assessment without a determination that the assessment followed the recommendations of the Advisory

1. For example, note the current ongoing work of President Clinton's Commission on Risk Assessment and Risk Management (scheduled to issue a report on April 24, 1996) and the ongoing work of the U.S. Environmental Protection Agency to issue Revised Carcinogen Risk Assessment Guidelines.

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Committee. Publishing an incomplete and unreviewed ETS assessment now has the potential of eclipsing the State's stated commitment to uniform, sound risk assessment practices.

- As developed more fully herein, Cal/EPA cannot take any action based on reliance upon U.S. EPA's Risk Assessment. Cal/EPA should not rely upon U.S. EPA's Risk Assessment on ETS, and in so doing, avoid the necessary and required original work by Cal/EPA. Under the California Health and Safety Code, 539660(c), Cal/EPA must evaluate the "quality of data" underlying any health evaluation. Cal/EPA cannot and should not pursue any report which relies upon EPA's Risk Assessment.

- Cal/EPA's ETS risk assessment (and specifically the lung cancer sub chapter) should not be pursued because Cal/EPA lacks the requisite authority to pursue such work. Cal/EPA's risk assessment on ETS was initiated prior to the enactment of AB 13, which imposed a state-wide ban on smoking in most places in California. See Labor Code sec. 6404.5(b). The restrictions of AB 13 encompass most nonresidential areas in the state, and even where smoking is restricted to designated areas, AB 13 requires ventilation equipment meeting specified standards to ensure that the air is not recirculated but exhausted outside. Even prior to AB 13, the Proposition 65

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Scientific Advisory Panel listed ETS as a carcinogen. As a result, in those areas where limited smoking is allowed, Proposition 65 requires that warnings be posted to alert persons of their potential exposure to ETS. Given the existence of AB 13 and Proposition 65, and given the authority of the California Occupational Safety and Health Administration to generally regulate worker health and safety it is difficult to see any basis of authority for Cal/EPA to pursue a risk assessment concerning ETS.

- Cal/EPA should decline to take any further action on the ETS risk assessment given the extensive regulatory work already underway at the U.S. Occupational Safety and Health Administration (OSHA). Not only does Cal/EPA lack authority to pursue an ETS risk assessment, but if there were a current compelling need to review ETS issues, that need is being met by U.S. OSHA. Federal OSHA has completed the public comment phase of the lengthiest, most extensive regulatory hearing and review of potential ETS effects ever conducted. The agency held six months of public hearings, amassed more than 110,000 comments from interested parties and scientific experts, and collected extensive studies and data regarding the claimed health effects of exposure to ETS. Unlike the brief Cal/EPA workshops, the federal OSHA hearings were conducted as part of federal OSHA's regulatory responsibility for determining

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- Reissue Chapter 7 of the Cal/EPA assessment in its entirety, as the new table of contents indicates some changes from the chapter on "other cancers" issued in May 1994.

- Substantially broaden the scope of its literature review to include all relevant materials. In particular, Cal/EPA should consider the public record compiled in response to U.S. OSHA's rulemaking on indoor air quality, which contains a vast amount of literature as well as perhaps the most recent and comprehensive analyses of ETS issues, many of which are cited herein.

- Given the unresolved criticisms of the U.S. EPA Risk Assessment on ETS, not rely on that document and its analyses, but obtain the relevant literature and re-analyze it. That is, Cal/EPA must "start from the beginning." In this regard, the agency should obtain and analyze the underlying data -- particularly the data from the Fontham, et al., study.

- Abandon the selective approach taken in the present draft of the Excerpt, and clearly define the criteria used in evaluating data and delineate the rationale for its approaches, in order to reduce the appearance of subjectivity.

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- Revise and reissue in draft all other chapters in this assessment of ETS risks, to incorporate the suggestions inherent in the foregoing as to all chapters.

- Review the Congressional Research Service Report. Most of Cal/EPA's overall risk assessment does not have the benefit of the November 1995 Congressional Research Service (CRS) report on ETS which raised serious questions about the methodology of the U.S. EPA's ETS risk assessment. The CRS study was not addressed in Cal/EPA's most recently released draft excerpt.

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SECTION II: CAL/EPA'S REVIEW IS SUBJECTIVE, SELECTIVE AND UNSCIENTIFIC

Cal/EPA's Excerpt clearly appears to be designed to support the conclusion that ETS exposure is associated with an increased lung cancer risk. The following discussion will illustrate a number of specific examples of subjectivity and selectivity in the Excerpt.

A. Cal/EPA Frequently Engages in Selective Reporting

Cal/EPA selectively references studies and reports data from studies in a manner that strengthens its positions. One example of this selectivity is found in the citation of the "first" studies to be published on spousal smoking and lung cancer. Cal/EPA cites only the Hirayama[1] and Trichopoulos, et al.,[2] studies, published in 1981. (p. 8) This statement is incomplete. Also in 1981, Garfinkel published data from a large cohort study conducted in the United States.[3] Unlike the Hirayama and Trichopoulos studies, Garfinkel's data support **no increased lung cancer risk associated with ETS exposure.** The failure to include the Garfinkel (1981) study -- whether intentional or an oversight -- gives the appearance that it was omitted because it did not support the theme being developed by Cal/EPA.

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Elsewhere, Cal/EPA claims that U.S. EPA's Risk Assessment on ETS is the "most recent" meta-analysis of the epidemiologic data on ETS exposure and lung cancer. (p. 8) This is inaccurate -- the results of several meta-analyses that fail to support U.S. EPA's conclusions have subsequently been published.[4-10] Again, the omission of these references contributes to the impression that Cal/EPA is not presenting a thorough, complete representation of the scientific literature, and is instead focusing only on material that supports its conclusions.

Cal/EPA is also guilty of selectively reporting data from individual epidemiologic studies. For example, in the discussion of childhood ETS exposure, Cal/EPA references a reported risk for "high exposure" during childhood of 2.07 (95% CI 1.16-3.68) from the Janerich, et al., study. This is the **only** statistically significant association reported in that study.[11] Not only could a single statistically significant risk estimate have arisen by chance, given the number of analyses reported in that paper, but taking that result out of context misrepresents the Janerich, et al., study. Cal/EPA could just as well have highlighted the workplace risk estimate from the Janerich, et al., study, 0.91 (95% CI 0.80-1.04) in the Excerpt's workplace discussion -- but it did not.

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Similarly, Cal/EPA selectively reports other data on workplace ETS exposures and lung cancer risk. Only two studies have reported workplace risk estimates that are statistically significant.[12,13] The vast majority of workplace risk estimates are not statistically significant.[11,14-25] While this is evident from the table of workplace results in the Excerpt, the text does not make this clear. Nor does the text indicate that recent meta-analyses of the workplace data report a summary risk estimate of approximately 1.0, consistent with no increase nor decrease in risk.[4,5]

Cal/EPA also engages in "data-dredging" in discussing the workplace data. Namely, it creates a subcategory of three studies "in which the assessment of workplace exposure to ETS was complete," (p. 18) and claims that those studies are "generally supportive of an association between workplace ETS exposure and risk of lung cancer." (p. 18) However, Table 7.7 of the Excerpt shows that only one of the three risk estimates, that of Fontham, et al., is statistically significant. The workplace risk estimates for the other two studies cited to support Cal/EPA's proposition (Wu, 1985, and Wu-Williams, et al., 1990) are not statistically significant, and thus do not clearly support an association, contrary to the Cal/EPA's claim.

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The review of workplace data by Cal/EPA appears to be an attempt by the agency to "make something out of nothing," because, as noted previously, meta-analyses of the workplace data consistently show no association.

Cal/EPA's discussion of the epidemiologic data on reported ETS exposures from sources other than the spouse is also flawed, because it relies in large part on only the reported results of the four most recent studies, rather than discussing the data presented in **all** the studies on this topic. As reported in a recent comprehensive review of the ETS-lung cancer literature, the data on lung cancer risks for non-spousal ETS exposures, taken as a whole, provide "little or no substantial evidence of an association."[26] Cal/EPA's approach to the data effectively ignores such analyses.

B. The Excerpt Contains Misleading Statements

There are a number of instances in the Excerpt where statements are framed in such a manner as to yield misleading impressions. Cal/EPA must draft its document so that its meaning is clear.

For instance, on p. 8, Cal/EPA states: "In order to gain a more accurate estimate of the association between ETS exposure

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and lung cancer, a meta-analysis approach has been used to pool results of **comparable** studies." (emphasis added) This statement implies that the spousal smoking studies are "comparable," when it has been clearly illustrated that they are not.[26] Moreover, it implies that meta-analysis may be appropriately applied to these studies, which is not a universally accepted position.[27]

On the same page, the Excerpt states: "Most of the individual studies found a small increased risk." (p. 8) Actually, of the 40 studies presently available,[1-3,11-18,20-25,28-30] only eight report statistically significantly increased overall risk estimates for spousal smoking and lung cancer.[1,2,13,18,25,35,41,42] The vast majority -- 80 percent -- of the spousal smoking studies report overall risk estimates that are not statistically significant. Without statistical significance, a reportedly increased risk is compatible with the null hypothesis of no association. Thus, Cal/EPA's reference to a "small increased risk" is misleading.

Another example of misleading reporting is found in Cal/EPA's treatment of the Fontham, et al., study. Cal/EPA states that cotinine measurements were obtained for 81 percent of self-respondent cases. (p. 15) The choice of this percentage is misleading. Fontham, et al. (1994), state: "Urine samples were analyzed for 356 (53.5%) of 665 cases and 1064 (83.3%) of 1278 controls."[13] Thus, cotinine samples were available for only about

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half of all the cases in the study, according to the original report. Here, Cal/EPA's choice of the value to cite seems to be intended to portray the Fontham, et al., study's methods in the most favorable light.

In another section discussing the Fontham, et al., study, Cal/EPA states: "[T]he U.S. multicenter study corroborated the subjects' self-reported current nonsmoking status using the urinary cotinine level." (p. 21) While Cal/EPA acknowledges that cotinine measurements assess **only** current smoking, it accepts the misclassification rates of 0.6 percent in cases and 2.3 percent in controls reported by Fontham, et al., as accurate. The Excerpt does not mention that Fontham, et al., measured cotinine in hospitalized cases. The vast majority of hospitals severely restrict smoking (in fact, accreditation requires that smoking be banned); moreover, many lung cancer patients who happen to be smokers stop smoking after diagnosis. Thus, the cotinine measurements in this study do not even give a good indication of present smoking status, let alone previous long-term smoking status. The smoking status misclassification rates portrayed by Fontham, et al., as accurate are, in reality, **not** representative of the true situation. As reported by Lee and Forey, a review of the literature indicates that smoking status misclassification rates may range as high as 15 to 20 percent.[51]

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C. **Cal/EPA Fails to Provide Justification for Considering the Recent Epidemiologic Studies Separately from the U.S. EPA Risk Assessment**

One of the most curious omissions in the Excerpt is its failure to discuss the outcome of a U.S. EPA-style meta-analysis in which data from the four epidemiologic studies published since 1991 are included. Cal/EPA provides no justification for this omission. Given Cal/EPA's acceptance (and even defense) of the U.S. EPA meta-analysis, the failure to consider a meta-analysis including all the studies can be interpreted as an attempt to exclude an analysis that would not support Cal/EPA's conclusions.

A review of the scientific literature reveals several post-EPA meta-analyses conducted by other researchers.[4,7-10] These meta-analyses illustrate that, if the U.S. EPA's approach is adopted, inclusion of new studies lowers the overall summary risk estimate.

For instance, in a recent paper, LeVois and Layard report:[4]

> Using the EPA's methods and assumptions, we have calculated a summary relative risk of 1.07 from a meta-analysis of 13 U.S. female spousal smoking studies, including these two recent studies [Brownson, et al., and Stockwell, et al.] This relative risk, with

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> 95% confidence interval of 0.95-1.21, is not statistically significant.

The inclusion of two additional recent studies in the meta-analysis effectively reversed the conclusion of the U.S. EPA Risk Assessment on ETS. The summary relative risk is no longer statistically significant, and, therefore, does not support a conclusion of an association between spousal smoking and lung cancer.

Gross (1995) noted that when Kabat (1990, an initial report of the study discussed in the Excerpt[52]), Stockwell, et al.,[22] and Brownson, et al.,[14] are included in a U.S. EPA-type meta-analysis, a summary risk estimate of 1.12 (95% CI 0.99-1.27) is calculated -- prior to adjustment for smoking status misclassification.[7] Gross states: "Since any such adjustment lowers the relative risk, it implies the null hypothesis of no association cannot be rejected." In another 1995 paper, Gross provides the misclassification-adjusted summary risk estimate; even when the U.S. EPA's very low rate of 1 percent is used, the summary value is 1.07 (95% CI 0.95-1.21).[8]

Similarly, Gori (1994) observes that, while he does not endorse the U.S. EPA approach:[9]

> Including the two latest studies [Brownson and Stockwell] explicitly excluded from the EPA's review and using the EPA's own [adjustment]

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> procedure, a realistic correction [for misclassification] of only 2.5% would nullify any excess risk estimates in the meta-analysis of the 13 available U.S. studies of spousal exposure.

Gori states that the published literature suggests smoking status misclassification rates between two and 10 percent (average four to five percent), compared to the 1.09 percent used by U.S. EPA.[9]

Sugita, et al., also reanalyzed the U.S. spousal smoking data, incorporating the Stockwell, et al., and Brownson, et al., studies, and calculated a summary risk estimate for the U.S. studies of 1.10 (95% CI 0.97-1.26).[10] Based on this and other summary risk estimates calculated by Sugita, et al., they conclude:

> From these odds ratios it has not been demonstrated scientifically whether or not ETS is a particular risk factor for lung cancer. The conclusion that the relationship between exposure to ETS and lung cancer is weak remains unchanged. (p. 180)

It seems clear that Cal/EPA facilitated its arguably predetermined conclusion by simply making observations about the "concordance" or "similarity" of the recent studies to U.S. EPA's conclusions, rather than by conducting its own statistical analysis. Cal/EPA should explain why the approach of comparing the individual post-1991 studies to the U.S. EPA summary risk estimate

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was chosen instead of incorporating the new studies into a meta-analysis or other, more formal, comparison.

Cal/EPA should also provide further explanation for its position that the recent studies support the U.S. EPA's conclusion, given that the Congressional Research Service reached the following conclusion, based on a review of the same material:[53]

> The new studies, including the very large Brownson study, did not clarify the existence of a risk. Indeed, they complicated the interpretation of the evidence, since the two largest U.S. studies -- Fontham and Brownson -- found in one case a positive risk that was barely statistically significant and the other no risk at all. (p. 25)

D. Subjective Terms Are Used Throughout the Excerpt Without Definition

Throughout the Excerpt, Cal/EPA employs terms like "concordance" (p. 7), "very similar to" (p. 7), "closest to" (p. 7), "consistency" (p. 27), "similar" (p. 14), and "compatible" (p. 19) when discussing the results of two or more studies or analyses. Because these terms are not defined, their scientific meaning is unknown to readers of the Excerpt, who are therefore left with the impression that these terms are subjective descriptions without a rigorous scientific basis.

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For example, Cal/EPA states: "Results from the largest population-based study, conducted in several metropolitan areas of the U.S. (Fontham et al., 1994) were **closest to** the pooled estimate of the U.S. EPA report." (p. 7) (emphasis added) The subjective phrase "closest to" has no scientific meaning. Used in this manner, it appears to be a transparent attempt to bolster the position that the newer studies support the conclusions of the U.S. EPA Risk Assessment, without conducting a statistical analysis.

E. The Excerpt Contains Unreferenced and Poorly Referenced Claims

In the Executive Summary of the Excerpt, Cal/EPA claims that there is "compelling biologic plausibility of an effect of ETS on lung cancer." (p. 7) No references are cited in support of this proposition. Thus, the public may take this statement at face value, when the statement is actually not supported by a review of the relevant scientific literature. Neither animal inhalation studies nor genotoxicity studies provide support for the claim, as discussed elsewhere in this submission (Section III).

Similarly, Cal/EPA provides only one reference for its claim that "there is no publication bias against statistically nonsignificant results on ETS in the peer-reviewed literature." (p. 10) The issue of publication bias cannot be summed up by a

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single reference; Cal/EPA should review additional literature on this issue, including the literature on meta-analysis, where much of the debate on publication bias has developed.

Moreover, Cal/EPA does not address the issues of selective reporting and "data-dredging," another form of bias common in epidemiologic studies. For instance, the Fontham, et al., study reports more than 100 case-control comparisons.[13] With this many comparisons, a number of statistically significant associations would arise by chance alone. This issue is not adequately addressed in the original studies, nor in the Excerpt.

F. Cal/EPA's Reanalysis of Kabat, et al. (1995), Is Not Justified

Cal/EPA's analysis of the 1995 Kabat, et al., case-control study[17] certainly gives the appearance of a bias toward reporting results that support the position that the recent ETS epidemiologic studies support the conclusions of the 1993 U.S. EPA Risk Assessment on ETS. In fact, the approach taken by Cal/EPA has already been deemed "controversial" by observers.[54]

The risk estimates based on the Kabat, et al., data, as originally reported in the publication, do not agree with Cal/EPA's position. As Kabat and colleagues stated: "[T]he pattern of odds

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ratios [in this study] shows little indication of an association of environmental tobacco smoke with lung cancer in nonsmokers."[17]

After noting that "[t]here were no significant associations between spouses' smoking and risk of lung cancer in male or female subjects" in the original report of the Kabat, et al., study, Cal/EPA states: **"We calculated the OR for lung cancer in males and females combined to be 1.19 (95% CI=0.76-1.87) in association with spousal ETS exposure."** (p. 18) (emphasis added) Cal/EPA then summarizes its conclusions: "Although [Kabat et al.] . . . interpreted their findings (analyzing men and women separately) to be unsupportive of an association between ETS exposure and risk of lung cancer, the odds ratio we calculated from their results, though not statistically significant, was in fact very similar to the pooled estimate from the U.S. EPA report." (p. 19)

Because the author gives no statistical or scientific justification for combining the Kabat, et al., data for men and women, this approach appears to be an attempt to "make the data fit." The decision to combine the male and female data is even more curious when one considers that the U.S. EPA Risk Assessment based its conclusions on data for women only, completely ignoring the available data on males, which, taken as a whole, support no increase in lung cancer risk in men whose wives smoked.[26] Moreover,

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the Excerpt implies that there is some importance in the reanalysis of the Kabat, et al., yielding a risk estimate of 1.19, the same as the U.S. EPA summary risk estimate, when this simple coincidence has little, if any, real meaning.

The interpretation by Kabat, et al., that their data do not support an association between ETS exposure and lung cancer risk is wholly appropriate. Kabat and colleagues report a number of risk estimates that do not achieve statistical significance. They also exercise caution in interpreting the few statistically significant risk estimates in their study. However, Cal/EPA does not simply accept this interpretation, apparently since it does not support Cal/EPA's position. Interestingly, Cal/EPA makes no comment about the unsupported interpretation by Brownson, et al., of the data in their case-control study.[14]

An examination of the reported results in the Brownson, et al., study shows that the overall risk estimate for spousal smoking is 1.0 (95% CI 0.8-1.2), clearly inconsistent with an increase in risk. The only statistically significant results in the Brownson paper were restricted to "qualitative" indices of exposure, where study subjects provided an "estimate [of] a perceived level" of exposure.[14] Despite the vast majority of the data being consistent with no increase in risk, Brownson, et al., concluded:

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> [O]ur study and others conducted during the past decade suggest a small but consistent elevation in the risk of lung cancer in nonsmokers due to passive smoking.

According to a scientist who has reviewed and reanalyzed the Brownson publication and the raw data from the study, "influences directed to the identification and reporting of an association between ETS [exposure] and lung cancer may have affected the reporting of the results" from this study and others.[55]

G. Conclusion

As the lung cancer Excerpt is written, it appears to be an attempt by Cal/EPA to make recent data agree with the conclusions of the U.S. EPA Risk Assessment on ETS. The selectivity and subjectivity of the Cal/EPA discussion lead to conclusions which simply are not supported by the data and objective, thorough scientific analyses.

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SECTION III: CAL/EPA RELIES INAPPROPRIATELY ON THE U.S. EPA RISK ASSESSMENT ON ETS, WITHOUT SUFFICIENT ATTENTION TO THE DEMONSTRATED FLAWS IN THAT DOCUMENT

Cal/EPA relies extensively on the 1993 U.S. EPA Risk Assessment on ETS, which purportedly reviewed the literature on ETS and lung cancer through 1991.[1] Cal/EPA has apparently accepted the U.S. EPA Risk Assessment as conclusive. While mention is made of certain criticisms of the U.S. EPA document (e.g., pp. 9-10), Cal/EPA simply lists those criticisms, without providing sufficient rebuttal. Cal/EPA sums up its discussion of the U.S. EPA Risk Assessment by stating: "[T]he U.S. EPA's report is based on the total weight of evidence, and not on an individual study." (p. 10) The following discussion will show that (i) the criticisms leveled against the U.S. EPA report are much more severe than the superficial treatment by Cal/EPA would imply, and (ii) U.S. EPA omitted a large amount of relevant data and information in reaching its conclusions about ETS, so that the "weight of the evidence" claim is misleading.

A. Criticisms of the U.S. EPA Risk Assessment Have Never Been Adequately Addressed by the Agency

Criticisms have been raised about the U.S. EPA's Risk Assessment since the initial release of a draft for public review in 1990. At that time, independent scientists and interested parties raised concerns about many important issues; over 100

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submissions were made to the U.S. EPA public comment docket. The numerous criticisms of the 1990 draft were summarized in a document prepared for the Agency, which stated:[2]

> The predominant theme in comments related to lung cancer is that the classification of ETS as a Group A carcinogen, causally related to increased risk of lung cancer, is unwarranted. . . . The claim that it is biologically plausible that ETS is a lung carcinogen is contentious and unjustified in the Report. (pp. 3-4)
>
> It is not possible to summarize comprehensively in just a few pages the remarks from the thousands of pages of comments submitted. (p. 4)

The criticisms -- on toxicological, chemistry, exposure, epidemiologic and statistical issues -- raised with respect to the 1990 draft of the U.S. EPA Risk Assessment were not adequately addressed in the revised draft that was issued in 1992. At that time, comments were again submitted by a number of independent scientists and other interested parties. In addition to reiterating many of the criticisms initially raised, and confirming that those criticisms were not adequately addressed in the new draft, the comments also raised new issues related to the second review draft.

Following the release of the final document in January 1993, essentially identical to the heavily criticized 1992 draft,

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a number of articles critical of the Risk Assessment have appeared in the scientific press.[3-9] For instance, Huber and colleagues (1993) wrote:[3]

> [T]he EPA's risk assessment is built on the manipulation of data, ignores critical chemical analyses and key epidemiological data, violates time-honored statistical principles, fails to control adequately for important confounding influences (factors other than the one studied that may affect a result or conclusion) that provide alternative explanations for its conclusions, and violates its own guidelines for assessing and establishing risk to a potential environmental toxin. It lacks credible quality control and adequate external unbiased peer review. **In short, in its report on ETS, the EPA did not comply with accepted principles of toxicology, chemistry, and epidemiology, nor with its own guidelines for undertaking cancer risk assessment. In fact, the conclusions drawn by the EPA are not even supported by the EPA's own statements.** (p. 45) (emphasis added)

In a 1994 commentary on the U.S. EPA Risk Assessment, Gori stated:[4]

> [T]his figure [3,000 deaths] is . . . the **result of an unprecedented exercise in data manipulation.** Among other unjustifiable gambits, this EPA report stands out for its unorthodox insistence on one-tailed statistics and 90% confidence intervals, for arbitrary and unproven adjustment procedures, and for its selective use of epidemiologic evidence. For instance, without explanation the report has summarily excluded workplace data and the latest epidemiologic studies, which together void the report's conclusions when added to the body of evidence. (emphasis added)

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Furthermore, in another discussion published in 1995, Gori observed:[5]

> EPA claims to have used a weight of evidence approach. In reality, ETS has been characterized only indirectly by an analogy to active smoking that EPA itself discounts. Negative epidemiologic reports were summarily ignored. Statistical standards were relaxed. Competing and well-known risk factors were disregarded. Documented misclassification biases were discounted. **By its own admission, EPA made very selective use of available studies, emphasizing only those that support its preconceived conclusions.** (pp. 20-21) (emphasis added)

In a technical analysis, Gross (1995) illustrated clearly that the U.S. EPA methodology for calculating deaths purportedly attributable to ETS exposure "is **far from an exact or even approximate science**. . . . [M]inor to moderate changes in the model parameters lead to large changes in the estimated number of [deaths]."[6] (p. 411) (emphasis added) Further, he stated that "EPA has not given very much thought to the uncertainty of its estimates," and noted that the epidemiologic data on ETS and lung cancer are consistent with a risk estimate of 1.0, which, in U.S. EPA's model, would yield an estimate of zero "attributable" deaths.

More recently, the Congressional Research Service (CRS) has issued two reports that raise substantial questions about the

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U.S. EPA Risk Assessment.[10,11] In 1994, one of the authors of the 1994 CRS review, Jane Gravelle, described the conclusions of that document as follows:[12]

> [O]ur evaluation was that **the statistical evidence does not appear to support a conclusion that there are substantial health effects of passive smoking.** (emphasis added)

A compilation of materials criticizing or questioning the U.S. EPA Risk Assessment is submitted as Appendix I to this comment.

B. Data on ETS Chemistry Were Omitted from the U.S. EPA Risk Assessment

The U.S. EPA Risk Assessment failed to discuss or even to reference a significant amount of relevant literature on the physical and chemical properties of ETS.

A number of scientists have concluded that ETS is not the same as either mainstream or sidestream smoke.[13-17] ETS is an aged and diluted mixture of sidestream and exhaled mainstream smoke; ETS is a dynamic, ever-changing mixture that undergoes chemical transformations and physical changes as it ages and is diluted in the air.[13-15] As one researcher in tobacco smoke chemistry has observed, "there are profound physical and quantitative chemical

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differences" among the three kinds of smoke (mainstream smoke, sidestream smoke and ETS).[13]

Studies also indicate that constituents in ETS are **hundreds to thousands** of times more dilute than either sidestream or mainstream smoke.[16,17] Concentrations of ETS constituents in real-life situations are often below the limits of detection and measurement for even the most sensitive air monitors. Often, the contributions of ETS constituents to the ambient air are indistinguishable from background levels of the same constituents generated by other sources.[16]

The strategy of comparing mainstream and sidestream smoke employed by U.S. EPA in the Risk Assessment (e.g., Chapter 3) ignores the profound effect of dilution in the ambient air upon tobacco smoke constituents. As two tobacco smoke chemists report:[14]

> The important question is not the ratio of sidestream/mainstream but rather what is the concentration of the constituent in the indoor environment and how does it compare to levels from sources other than ETS. Studies based solely on observations of fresh sidestream, or highly and unrealistically concentrated ETS should take into account the possible differences between these smokes and ETS found in real life situations.

Similarly, the 1986 Report of the Surgeon General notes: "SS [sidestream smoke] characteristics, as measured in chambers, do not

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represent those of ETS, as inhaled by the nonsmoker under non-experimental conditions."[18]

C. Data on Measurements of ETS Exposure Were Omitted from the U.S. EPA Risk Assessment

Cal/EPA focuses heavily on the epidemiologic literature in the Excerpt. In failing to mention the literature on issues of ETS exposure, Cal/EPA seems to be implying that the U.S. EPA Risk Assessment provides sufficient discussion of these issues, which is not the case.

The initial public review draft for the U.S. EPA Risk Assessment (1990) did not even contain an exposure assessment. Comments submitted to the public docket for the 1990 draft risk assessment observed that U.S. EPA had failed to provide an exposure assessment that considered data from the numerous published studies on actual levels of ETS constituents in the air of public places and workplaces. In apparent response to that criticism, the revised 1992 draft of the U.S. EPA Risk Assessment contained a chapter entitled "Estimation of Environmental Tobacco Smoke Exposure," but the chapter's authors failed to consider at least 35 pertinent exposure studies on ETS constituent levels in public places.

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Furthermore, during its review of the chapter in July of 1992, the U.S. EPA Science Advisory Board rejected the chapter and returned it to the author for rewriting. Nevertheless, without either an exposure assessment or recourse to any of the data pertaining to it, the Science Advisory Board endorsed U.S. EPA's estimate of exposure and risk for the entire U.S. population. A revised chapter on ETS exposure occurs in the final U.S. EPA Risk Assessment; however, the studies and data therein are not integrated into the U.S. EPA Risk Assessment.

Only a small number of actual ETS exposure studies available in the published literature are even discussed in the U.S. EPA Risk Assessment. Many of the studies of exposure to ETS constituents under realistic conditions in public places, workplaces and homes omitted from the U.S. EPA Risk Assessment report minimal exposures to ETS; these reported exposures do not support the conclusions of the U.S. EPA Risk Assessment.[17,19-62]

For instance, studies on ambient measures of nicotine in offices and restaurants report average levels of approximately 2.0 and 3.5 ug/m^3 nicotine, respectively. These exposures are equivalent to 1/400 to 1/200 of the nicotine found in a single cigarette. Averages for nonsmoking areas in workplaces with smoking restrictions are even lower, averaging less than 1 ug/m^3 nicotine, or about 1/1,000 of the nicotine in a single cigarette.

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This means that the typical nonsmoking worker would have to spend from 200 to more than 1,000 hours in an office, restaurant or public place in order to be exposed to the nicotine equivalent of a single cigarette.[35,55-58]

The cigarette equivalent approach has been criticized because different results would be obtained if different reference compounds are chosen. These criticisms are based simply on calculating the sidestream/mainstream ratios for the different reference compounds. Such a calculation would be accurate if there were no chemical and physical changes which occur when sidestream smoke is transformed into ETS. This, as has already been clearly pointed out earlier in this submission, is not the case. As a consequence, it would not be at all correct to state that a typical nonsmoking worker who spends 200 to 1,000 hours in an office, restaurant or public place is exposed to the **smoke** equivalent of one cigarette, since ETS is not at all chemically or physically identical to the smoke to which a smoker is exposed.

With regard to the calculation of cigarette equivalents based on other reference compounds, it is important to note that the vast majority of ETS constituents are found, generally in much greater amounts, in indoor environments in the absence of ETS. Researchers report that there is little difference in ambient levels of carbon monoxide or nitrogen oxides in smoking and

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nonsmoking areas of workplaces and public places and in homes with or without smokers.[16,27,30,31,49,59,60] Similarly, levels of volatile organic compounds such as formaldehyde and benzene in the presence of smoking are often indistinguishable from levels reported in nonsmoking areas.[16,32,35,50-52] Studies that have examined ETS constituent levels of nitrosamines also report minimal contributions to overall ambient air levels in homes, offices and public places.[48,61,62]

D. Limitations of Exposure Estimates Based on Questionnaires Were Not Discussed by U.S. EPA

The "exposure" estimates in epidemiologic studies on ETS are wholly independent of a large body of data on ambient exposure measurements to various constituents of ETS, which, taken as a whole, suggests that ETS exposures are minimal. The epidemiologic studies rely on questionnaire data, the accuracy of which depends on an individual's ability to recall past events, such as how much a husband smoked in the past 20 to 30 years.[63-65] The two bodies of data, epidemiologic/health effects, on the one hand, and exposure measurements on the other, have never been integrated into one comprehensive study.

In a 1994 article critical of the U.S. EPA Risk Assessment, Gori wrote:

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> A major limitation of epidemiologic studies on ETS has been the unreliable estimates of dose, which compound the uncertainties of personal or proxy recall of the intensity, frequency, and duration of exposures over individual lifetimes. Even the simple dichotomous classification of exposed and non-exposed subjects presents recognized uncertainties, such as those deriving from the self-classification of some smokers as non-smokers. (p. 327)

U.S. EPA relied heavily on the epidemiologic data in its Risk Assessment on ETS, without adequately exploring the limitations of questionnaire data.

1. "Spousal Smoking" Is Not Equivalent to ETS Exposure

A fundamental problem with Cal/EPA's Excerpt and with U.S. EPA's Risk Assessment is the **uncritical** acceptance of the risks reported in spousal smoking studies as **true** risks due to **exposure to ETS**. The central question is whether or not "ETS exposure" is the same thing as "spousal smoking"; that is, does living with a smoker imply exposure to ETS?

Even though U.S. EPA concedes that spousal smoking status is a "crude measure" of ETS exposure and one that is "prone to exposure misclassification,"[66] the Agency nevertheless equates spousal smoking status with household exposures to ETS.[67] However, the extent to which the spousal smoking index is an accurate,

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reliable and valid quantitative indicator of ETS exposure was not assessed by U.S. EPA in its Risk Assessment. The quality of the underlying exposure data from the spousal smoking studies is thus of critical importance for epidemiologic studies on ETS and chronic disease, because "the accuracy of any statistical analysis is limited by the accuracy of the information upon which it is based."[68]

The spousal smoking index is not an accurate marker for exposure to ETS. That "marriage to a smoker" is **not** equivalent to home exposures to ETS has been demonstrated in two studies.[69,70] Friedman, et al., polled nearly 38,000 nonsmokers and ex-smokers in California about ETS exposure. Nearly 35 percent of respondents classified by their spouse's smoking status reported no exposure overall to ETS; 47 percent of women married to smokers reported zero hours of exposure at home.[69] The researchers concluded that "using the spouse's smoking status to classify persons resulted, as far as can be discovered with our relatively crude questionnaire, in a considerable amount of [exposure] misclassification."[69]

A 1994 published study conducted in the U.K. reports a similar conclusion.[70] The researchers compared subjective estimates of ETS exposure with objective measurements of airborne ETS markers via personal monitors (breathing zone measurements). They reported that 45 percent of subjects with a smoking partner assessed their

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exposure as "none or low." Similarly, 30 to 40 percent of individuals married to smokers were actually exposed to less than the average ETS levels measured among subjects with a nonsmoking partner. The researchers conclude: "Clearly, spousal smoking status would not be a reliable means to assess the ETS exposure of individuals or small groups of subjects."[70]

Thus, "marriage to a smoker" may -- or may not -- imply exposure to ETS; it cannot serve as an accurate, quantitative surrogate for actual exposure measurements.

2. Attempts to "Refine" the Spousal Smoking Index Do Not Yield Better Data

In attempting to "refine" the spousal smoking index, a number of spousal smoking studies have employed exposure estimates beyond the simple index of "marriage to a smoker."[65,71] Questions regarding ETS exposures have included those related to the **intensity** of exposure (e.g., the number of smokers in the home or the number of cigarettes smoked per day or per year), or the **duration** of exposure (e.g., hours of exposure, years of marriage to a smoker or years of smoke exposure).[71] Estimates of the intensity or duration of spousal smoking exposure are derived by questionnaire or interview of the study subject (the case) or next of kin.[72]

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There is no standardized or validated questionnaire available for use in epidemiologic studies on ETS, and no single questionnaire was used in the published studies on spousal smoking.[73] As a consequence, definitions of various ETS exposure indices have differed considerably from study to study with respect to source, intensity and duration.

3. Questionnaire Responses Are Neither Reliable Nor Accurate

The questionnaire, by its very nature, can provide only a crude, qualitative estimate of exposure. Questionnaire data do not, and cannot, provide information on **concentration** (e.g., actual levels of airborne ETS constituents) or **frequency** of ETS exposure. As the U.S. National Academy of Sciences observed in 1991: "Exposures [to ETS] occur at a wide range of concentrations for highly variable periods and in numerous indoor environments. Unlike active smoking, exposure to ETS cannot now be easily assessed. . . ."[73] The questionnaire is thus only an indirect means of assessing exposure. This, according to a NIOSH witness at the U.S. OSHA Public Hearing, is one of "the principal weaknesses in the epidemiologic evidence to date."[74]

Where exposure is defined as "concentration over time" or "intensity, frequency and duration," the questionnaire's inherent shortcomings are obvious. Thus, Coultas, et al., note:[75]

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> Questionnaires on exposure to environmental tobacco smoke generally assess the strength of the source, e.g., the number of smokers, the number of cigarettes consumed and the duration of exposure. The concentration of environmental tobacco smoke, however, depends not only on the source strength, but on room size, mixing, adsorption of smoke components, and the rate of exchange of indoor with outdoor air. Personal exposure also varies with the nonsmoker's proximity to the smoker. Questionnaires cannot comprehensively and accurately assess each of these factors.

Estimates of "intensity" and "duration" of exposure also depend upon respondent memories of literally decades of potential exposure scenarios. Complete recall, of course, is impossible, and even partial recall may be faulty. For example, studies indicate that while spouses (or children) may generally agree in reporting a partner's (or parent's) smoking status (i.e., whether or not a spouse smoked), agreement regarding the amount smoked or duration of smoking is often very poor.[63,65,76-79]

Pron, et al., examined the reliability of self-reported ETS exposure histories by interviewing and re-interviewing study subjects.[65] Consistency of responses about exposure between initial interview and re-interview was poor, and correlations between responses were low, especially for questions related to intensity and duration of exposure. The authors conclude:

> [T]his is the first study to assess the reliability of information reported on passive smoke exposures in personal interviews. Test-

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retest estimates of reliability suggest that misclassification of such exposure may be extensive.

In "An Assessment of the Validity of Questionnaire Responses Provided by a Surviving Spouse,"[76] Lerchen and Samet report that wives generally agree with the smoking status classification given by their spouse, but that 44 percent of the spouses could not provide a detailed smoking history of the spouse. The authors observed that wives tended to report that their spouse smoked 20 cigarettes per day, even when the husbands reported that they smoked substantially more or less. The researchers remarked that "the validity of such surrogate information, when available, is uncertain." Kolonel, et al., reported similar results in 1977.[80]

Sandler and Shore investigated the degree of agreement on parental smoking status from interviews of parents and their (adult) children.[77] These authors reported reasonable agreement in responses about the smoking status of the parent, but agreement on exposure level estimates was extremely poor. The authors conclude that childhood ETS exposure information obtained via questionnaire from adults "cannot readily be used to estimate levels of exposure" to ETS, and that "data on levels of exposure were not of high enough quality to allow for detailed evaluation of dose-response."[77]

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4. Questionnaire Responses Are Influenced by Perceptions

Reconstruction of a life history of ETS exposure is highly influenced by recall accuracy -- imagine recalling, with **any** degree of accuracy, the duration, frequency and degree of personal ETS exposure over the previous week, year or decade, or during a marriage, during adulthood, etc. Studies suggest that even **recent perceptions** of ETS exposure may be wholly inadequate indicators of **actual** ETS exposures.[64,70,81-83]

O'Connor, et al., compared self-reported exposures to ETS with ambient levels of nicotine collected by personal monitoring devices.[81] Thirty-six percent of women who reported ETS exposure by questionnaire were misclassified as "exposed" to ETS according to objective measures of exposure.

Coultas, et al., in a study of 10 homes, found that questionnaire responses about ETS exposures "were poor predictors of concentrations of respirable particles and nicotine."[64] Similarly, a workplace study conducted by Schenker, et al., reported no association between respirable nicotine concentrations and self-reported exposures to ETS.[82] The authors conclude that "self-reported exposure to ETS is an inaccurate measure of passive smoking in the occupational setting."

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Two recent, large-scale studies in the U.S. and the U.K. compared perceptions of individual exposures to ETS (by self-report) with actual levels of ETS constituents collected in the subjects' breathing zones by personal monitors in agreement with OSHA practice.[70,83] In the U.S. study, greater than 50 percent of all self-reports of ETS exposures occurred at work. Actual exposure measurements, however, indicated that average exposure levels were approximately four to six times greater in venues **outside** the workplace.[83] In the U.K. study, individuals subjectively ranked relative contributions of ETS as leisure > work > home > travel.[70] Measured exposure levels, however, indicated a ranking of: home > leisure > work > travel. The authors suggest that the discrepancy between subjective rankings and objective measurements of exposure may be due to the subject's inaccurate estimate of the time spent in each venue. Other researchers have studied variations in subjective perceptions of exposure and have reported that perceptions will vary depending or whether or not the exposure source (i.e., the smoker) can be seen.[84]

5. Cotinine Measurements Do Not Validate Questionnaire Responses

Nicotine, because it is characteristic of tobacco smoke in the air, has been used extensively as an ambient air exposure marker for ETS.[15,70,83,85] Likewise, cotinine, one of the substances converted from nicotine by the body, has been used as a biomarker

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(internal dose marker) for nicotine (and ETS) exposures.[26-30] Several researchers, including Riboli, et al., of IARC, have used measurements of cotinine in an attempt to validate self-reports of ETS exposures.[87,90-92]

The advantage of body fluid measurements of cotinine over nicotine is that cotinine has a longer half-life (17 hours vs. 3 hours). Despite this longer half-life, however, cotinine-derived estimates are of little value in determining past (greater than one week) exposures to nicotine. As Reasor has written:[33]

> At present, however, there is no reliable way, through the use of biological markers, to assess long-term exposure to ETS.

Cotinine has even more fundamental problems when used as a biomarker for exposure to ETS.[81,88,93-97] For example, an individual will metabolize and clear cotinine from his/her system at different rates at different times (intra-individual variation), and clearance rates vary considerably from individual to individual (inter-individual variation).[93,95,98] Common foods also contribute trace levels of nicotine (which are thus converted to cotinine), thereby confounding inferences about ambient nicotine (ETS) exposures.[99] Moreover, body fluid levels of cotinine do not correlate well with ambient levels of nicotine (or any other constituent of ETS),[64,70,81,91,96] and saliva, plasma and urine levels of cotinine are also poorly correlated.[64,95]

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Large intra- and inter-subject variabilities in the conversion, metabolism and clearance of cotinine call into question the validity of its use in surveys that employ single-point measurements (of cotinine) to represent ETS dose.[100] Dr. Paul Nelson of R.J. Reynolds recently observed: "It is likely that inter-individual variations in nicotine and cotinine metabolism or excretion would far outweigh the small incremental increase in cotinine concentration following exposure to typical levels of ETS nicotine. In other words, the variation between people is larger than the variation due to normal exposures."[101]

Virtually every cotinine measurement survey relied upon by U.S. EPA for ETS exposure estimates was restricted to single-point measurements of cotinine.[86,87,91,92] However, an individual's cotinine level at a single point in time will be determined by the timing of the specimen collection, and by the individual's own rates of uptake, metabolism and clearance. Thus, Idle observes:[95]

> Single point cotinine concentrations can give no more than a clue of past exposure to pyridine alkaloids [such as nicotine] of unknown amounts, at an unspecified time, by an unknown route of entry and from unknown origins.

Phillips, et al., reported extremely poor correlations between salivary cotinine values and 24-hour respirable particle and nicotine exposure measurements.[70] The data from the Phillips,

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et al., study indicate that some subjects who were exposed to high levels of particles and nicotine had **no** detectable cotinine levels, while some subjects exposed to low levels of ETS, as determined by personal monitor, exhibited high levels of cotinine.

O'Connor, et al., reported levels of urinary cotinine that "did not differ" among ETS exposed and non-exposed.[81] Ogden, et al., reported "virtually identical median levels of salivary cotinine" for all subjects, even though nicotine exposures varied nearly three-fold between exposed and non-exposed individuals.[96]

Cotinine measurements likewise do not correlate well with self-reports of ETS exposures.[70,75,80,87,102,103] Wagenknecht, et al., found that 58 percent of 575 study participants who reported ETS exposures of 42 hours or more had **no** detectable serum cotinine levels; of the 186 individuals who reported no known exposures, 23 percent had a detectable cotinine level.[102] Delfino, et al., examined salivary cotinine levels and compared them with questionnaire-derived responses about ETS exposures.[103] No correlations were reported.

In 1994, Emmons, et al., reported that nearly half of those individuals who recalled exposure to ETS at work had nondetectable cotinine concentrations, as did 29 percent of those who reported exposures at home.[87] Coultas, et al., found that both

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urinary and salivary cotinine levels of workers, measured post-workshift, "varied widely with self-reported exposures."[75] Similar results were reported by Heller, et al.,[94] and Suadicani and co-workers.[104] In the latter study, investigators reported that individuals who were classified (by self-report) as "often exposed," "occasionally exposed" and "rarely/never exposed" exhibited similar average serum cotinine levels of 25, 22, and 24 ng/ml, respectively.[104] Tunstall-Pedoe and coworkers recently reported poor correlations between self-reported ETS exposure and serum cotinine level.[89] They concluded that "their poor correlation with each other . . . undermine[s] the validity of the two measures of passive smoking."

Despite acknowledged shortcomings in the use of cotinine as a quantitative biological marker for ETS, some researchers nevertheless have used cotinine measurements in an effort to validate self-reports of ETS exposure.[86,91] Riboli, et al., for example, reported that mean urinary cotinine levels among their study population showed a linear increase with self-reports of ETS exposure, and that cotinine levels were indicative of reports concerning the duration of exposure and the number of cigarettes smoked in the presence of the subject.[91]

Close examination of the Riboli, et al., study data reveals, however, that 20 percent of the study population had

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nondetectable cotinine levels, and that a predicted increase of 5 ng/ml urinary cotinine could be calculated from self-reported exposures ranging anywhere from six to 83 cigarettes per day.[91]

Riboli, et al., do not indicate the existence of any overlap in cotinine measures among the groups examined.[91] Other researchers, however, have reported wide variations of cotinine measures within specific levels of reported exposure.[63,70,87] For example, Phillips, et al., reported "considerable variation in the direct measurements [of cotinine] corresponding to the higher grades of subjective [ETS] assessment."[70]

Even proponents of the use of cotinine to validate self-reports of ETS exposures realize the limitations of the method.[63] Cummings, et al., provide the following caveats to their study:[63]

> Cotinine was chosen as a biological marker of ETS exposure because it is specific to tobacco smoke. However, cotinine levels in body fluids may not only reflect environmental exposure to tobacco smoke, but also factors that influence uptake and metabolism of nicotine.

And:

> The relatively modest correlation between reported ETS exposure and urinary cotinine indicates that other factors such as differing metabolic rates and body size may have a confounding effect on the relationship between cotinine levels and questionnaire measures of ETS exposure. In view of this finding, we

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would recommend against using cotinine levels as a strictly quantitative indicator of ETS.

6. A Potential Bias in ETS Questionnaire Data

Self- (and surrogate) reports of exposure to ETS are neither accurate nor reliable when compared to a standard of personal measures of exposure to airborne ETS constituents. Questionnaires are limited by accuracy of recall and the individual's ability to provide comprehensive, quantitative estimates of exposure over time. Other problems beset the questionnaires used in studies on ETS. As Tager notes:[105]

> Among the most significant limitations of existing questionnaire data [on ETS] are the facts that many of the questions were not designed specifically to investigate involuntary exposure, or the questionnaires have been incomplete in their probing of the circumstances of exposure (e.g. intensity, duration, specific location, etc.). These limitations have made it difficult to provide even semi-quantitative exposure estimates over time.

The accuracy of an exposure estimate is of obvious importance because of its profound effect on risk estimates in epidemiologic studies.[106] Garfinkel, et al., in a spousal smoking study published in 1985, reported different risk estimates ranging from 0.46 to 3.57 depending upon who responded to questions about exposure. If a nonsmoking wife or smoking spouse responded to the

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exposure questions, the estimated risk from spousal smoking was below unity, i.e., no increased risk. If a son or daughter responded, the estimated risk rose to over 3.00. Responses were also influenced by changing the wording of the exposure-related questions.[106]

An entire range of potential biases is introduced through the improper design and administration of specific questionnaires. One particular bias, however, is directly applicable to the ETS exposure issue. It is one that arises, in part, from the way in which ETS exposure questions are phrased and presented -- called "recall bias."[72,107-109] Recall bias in relation to ETS is a differential bias, in that cases and controls are likely to be affected differently.[72]

Given the tremendous publicity generated, for example, by the U.S. EPA's classification of ETS as a "known human" carcinogen, it is without question that a nonsmoking lung cancer case could be influenced by such publicity in the effort to account for his/her disease. If ETS is mentioned or prompted in any way by the questionnaire or interviewer, the likelihood increases that ETS will be selected by the respondent as "the correct answer."[107] A control, or someone without lung cancer, is not likely to respond in that way. Tunstall-Pedoe, et al., for example, found that self-reports of exposure exhibited strong associations with symptoms,

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while the relationships were "weak or absent" for cotinine levels.[89] The authors suggest that individuals with respiratory conditions may exaggerate ETS exposures, thereby creating a (recall) bias in self-reported exposure estimates.

The influence upon the respondent by the way a question is phrased or asked may be extensive. It reflects, in Wynder's words, a "wish bias," a "tendency on the part of the subject or the investigator to reach a desired result."[137] Wynder writes:

> Research workers, like everyone else, often develop an affection for their own hypotheses and may prefer to see them supported rather than refuted. This may lead to incomplete review of the literature in which papers failing to support the hypothesis may be ignored or more subtly, may be rejected because they are considered to be of worse quality than papers that support it. Sometimes hypotheses are based on a single piece of evidence . . . The wishes of the investigator may also enter into the collection of the data. Greater care and thoroughness may be given to collection of the data from the cases than from the controls.

The foregoing analysis demonstrates that ETS exposure and the surrogate "spousal smoking history" are not the same thing. A spouse living with a smoker may or may not be exposed at home, and he or she may or may not be exposed elsewhere. If an individual is truly exposed to ETS, the questionnaire response provides no information on the concentration or frequency of exposure. Thus,

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if meaningful at all, the surrogate "spousal smoking" may be more of a measure of the many lifestyle factors surrounding marriage to a smoker than it is of ETS exposure; indeed, a history of spousal smoking may have a number of implications for disease that have nothing whatsoever to do with exposure to ETS.[110]

E. Relevant Animal Inhalation Studies Were Essentially Overlooked in the U.S. EPA Risk Assessment

U.S. EPA's risk assessment overlooked the vast majority of relevant animal data,[1] citing only a few studies. This omission severely undermines the claim that the U.S. EPA's conclusions are based on the "weight of the evidence." As the following discussion will show, if Cal/EPA were to review all the relevant animal data, it would be clear that those data provide no support for a claim that ETS exposure is associated with lung cancer.

The data in animal inhalation studies reported to date provide no support for a claim that there is "biological plausibility" for the position that ETS exposure is causally related to lung cancer. These data are not referenced in the Excerpt.

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The "carcinogenic agents" supposedly identified in tobacco smoke (e.g., the "list" of suspected carcinogens referred to in the U.S. EPA Risk Assessment) either are not suspected **pulmonary** carcinogens or have not been unequivocally demonstrated as tumorigenic to human tissue or to the lung tissue of experimental animals.[13,111,112]

In conjunction with U.S. OSHA's lengthy rulemaking process (which is still underway), several reviewers have examined and summarized the available animal inhalation studies relevant to U.S. OSHA's claim that animal inhalation studies provided supporting data for an ETS-lung cancer relationship. These reviewers unanimously conclude that the animal studies do not support the claimed carcinogenicity of ETS.[113-117]

One reviewer, Christopher R.E. Coggins, Ph.D., concluded that animal studies using close surrogates for ETS "show no meaningful toxicological changes, even at massive exaggerations of real-world ETS concentrations."[113] Similarly, Gordon Newell, Ph.D., told U.S. OSHA that studies using a number of animal species have "failed to support the hypothesis that fresh tobacco smoke causes lung cancer" in those species.[114]

Moreover, Gio B. Gori, Sc.D., noted in his submission to U.S. OSHA's rulemaking record:[115]

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> Experimental data offer no plausible argument to classify ETS as a human risk . . . The arbitrariness of a priori assumptions of ETS-related human risks is further underscored by equivocal and uninterpretable epidemiologic reports.

Overall, tobacco smoke inhalation studies have not produced an increased incidence of lung tumors in experimental animals compared to controls; the relevance of other routes of exposure (e.g., skin painting) is questionable.[116,117] The vast majority of the available data from animal inhalation studies using surrogates for ETS deal with subchronic exposures, which are of minimal relevance to the question of the claimed "carcinogenicity" of ETS. Nevertheless, none of the subchronic studies report data supporting any permanent changes following subchronic exposure of animals to sidestream smoke at levels exceeding those encountered in "real-life" situations. Brief summaries of the subchronic studies follow.

In two 1987 papers, Haley, et al., present preliminary reports on an American Health Foundation study, in which hamsters were exposed to mainstream or sidestream smoke 7 days/week for 18 months.[118,119] Apparently, however, no final report has been published. In those reports, the authors note that smoke-exposed animals were living **longer** than were sham or cage control animals. No additional information was presented.

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von Meyerinck and colleagues (1989) describe a study in which rats and hamsters of both sexes were exposed to sidestream smoke at a concentration of 4 mg/m^3 TPM and 25 to 30 ppm carbon monoxide for 10 hours/day, five days/week for 90 days.[120] These authors note about their exposure system: "The levels in the exposure chamber were at least 1 and in some instances 2 orders of magnitude higher than reported for smoke-polluted rooms under real-life conditions." (Elsewhere, the authors described these conditions as "unrealistically high."[121]) One hundred animals of each species were exposed, 115 of each species were sham controls, and 100 of each species were room controls. The authors reported minor, completely reversible histopathological changes in the nasal cavity in rats only, and no alterations in any other part of the respiratory tract.

Male rats and male hamsters were nose-only exposed to fresh sidestream smoke (FSS) for seven hours/day, seven days/week for 90 days, in a study reported in 1994 by Teredesai and Pruehs.[122] One group of 20 animals was exposed to FSS with a total particulate matter (TPM) concentration of 2 ug/L, one to FSS with TPM of 6 ug/L, and one served as a sham exposure group. Histopathological changes described as "slight" were reported in the nose and larynx of exposed rats, "mainly in the high FSS concentration group." These changes were reversible following cessation of exposure. The

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authors noted that "[n]o smoke-exposure-related histopathological changes were observed in trachea and lungs."

In the subchronic study of Coggins, et al.,[123] aged and diluted sidestream smoke was used as a surrogate for ETS. This substance may be a more appropriate approximation of ETS than are other forms of tobacco smoke. Effects (hyperplasia and inflammation) were reported only in animals exposed to particle concentrations some 100 times higher than typical real-world concentrations. Coggins, et al.,[124] also report the same minor, completely reversible histopathological changes. The changes did not progress over longer periods of exposure, and once again occurred only at particle concentrations some 100-fold higher than real-world levels.

In a 14-day inhalation study, one would not expect lung tumors to develop. Thus, the relevance of the work of Coggins, et al., to the discussion of cancer is limited. Nevertheless, Coggins, et al. show only minor, reversible cellular changes following intense exposure to a surrogate for ETS.[125]

Only one animal inhalation study to date has used exposures to a surrogate for ETS of a duration sufficient for it to be considered a chronic study.[126] Witschi, et al., recently reported the results of a study, specifically designed to produce

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lung tumors in the strain of mouse used, which failed to show any significant difference between unexposed animals and animals exposed to a surrogate for ETS. The strain used lives less than one year and is known to develop lung tumors within four to six months after the beginning of exposure to various chemicals.

In the Witschi, et al., study, these mice were exposed (whole-body) to concentrations of aged sidestream smoke well in excess of measured "real-world" levels. (While not equivalent to ETS, sidestream smoke has been used as a surrogate in animal studies.) Despite the demonstrated sensitivity of this strain of mouse, the authors reported the following: "The number of animals bearing lung tumors was the same in smoke-exposed as in filtered air-exposed animals as was the average number of tumors per lung." These data are compatible with the conclusion that sidestream smoke, under the test conditions, is not a lung carcinogen in this strain.

In a recent review of the relevant literature, Rodgman cautions:[13]

> Classifying a substance as tumorigenic or 'carcinogenic' can be misleading. Often, these terms are overinterpreted. One must be aware of the precise meaning and limitations of the terms tumorigenicity and carcinogenicity when applied to specific

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compounds and must exercise considerable care in the use of these and related terms.

* * *

Many of these 43 MS and/or tobacco components [claimed to be carcinogens] should be excluded from the list on the basis of published data on their tumorigenicity (or lack of it) in laboratory animals at levels determined in MS, their lack of tumorigenicity in most instances on inhalation, and the equivocal evidence of their tumorigenicity in humans at levels in MS.

In this major review, Rodgman also writes:

[I]nhalation studies from 1936 to date involving lifetime exposure of laboratory animals to whole cigarette MS have consistently failed to induce squamous cell carcinoma . . .

The failure to produce in MS-exposed laboratory animals the tumor type reported to be associated with smoking in humans is important not only with regards to the biological properties of MS itself but also with respect to that of diluted MS delivered to the caged animals. . . .

If, as Stewart and Herrold (1962) noted, these smoke-inhalation experiments more closely resembled passive smoke (or ETS) exposure than human exposure during actual smoking, then substantial evidence is available to demonstrate that exposure to 'passive smoke' (or ETS), more concentrated than that encountered in the human situation, is ineffective in induction of the tumor type supposedly associated with cigarette smoking in humans . . .

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The above discussion illustrates that the available animal inhalation data provide no support for the claimed "biological plausibility" that ETS exposure is associated with an increased lung cancer risk. The unreferenced statement made in the Excerpt is not representative of the actual state of the scientific literature, and as such, results in a misleading impression being conveyed to readers of the Excerpt. Cal/EPA should review the relevant animal inhalation studies and the critiques/discussions already in the U.S. OSHA rulemaking record as it revises this Excerpt.

F. Data on Genotoxicity and Related Endpoints Were Essentially Overlooked in the U.S. EPA Risk Assessment

The U.S. EPA Risk Assessment fails to reference a number of actual studies comparing levels of mutagens and other genotoxic markers in the body fluids of exposed and non-exposed nonsmokers.[127-136] The results of those studies suggest no statistically significant increases in mutagenic activity in the body fluids of nonsmokers exposed to realistic levels of ETS compared with nonsmokers who are not exposed.

When considering data on genotoxicity, it is important to put the genetic changes reported in such studies into context. All forms of life are constantly exposed to physical and chemical

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agents in the environment (e.g., radiation) and to endogenous (internal) agents with the ability to cause changes in DNA. According to Bruce Ames, developer of the Ames assay for mutagenicity, human exposure to potentially mutagenic or carcinogenic substances is much greater than generally appreciated, i.e., the environment can be thought of as "filled with potential carcinogens."[137] DNA has been called an "unstable" molecule, and it has been noted that **endogenous** DNA damage may occur at the rate of 100,000 base pairs **per cell, per day.**[137,138] Thus, DNA is **not** completely stable; changes are regularly occurring, but for the most part, do not result in heritable effects on the organism. As toxicologist Christopher R.E. Coggins of R.J. Reynolds stated in testimony before U.S. OSHA, "Toxicologically, I'm not sure that we really know what mutagenesis really means because of . . . DNA repair."[139]

Therefore, conclusions about genotoxicity obtained from <u>in vitro</u> systems, while certainly providing some information about the substance being tested, must nevertheless be put into the proper biological context. The magnitude of a genotoxic response in the whole organism may be substantially different than that observed in a bioassay.[140,141] As Ames and Gold noted:[140]

> [H]umans have numerous inducible defense systems against mutagenic carcinogens, such as DNA repair, antioxidant defenses, glutathione

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> transferases, and so forth . . . [L]ow doses of carcinogens appear to be both much more common and less hazardous than is generally thought.

The difficulty of extrapolating from *in vitro* genotoxicity to *in vivo* carcinogenicity is illustrated by data presented in the recent animal inhalation study of Witschi, et al., which used sidestream smoke.[126] Other studies suggest that tobacco smoke condensates may be mutagenic when tested using *in vitro* systems; however, there are no such studies using ETS condensates. In their recently published chronic inhalation study, Witschi and colleagues reported no differences in the total number of animals with tumors or in the average number of tumors per lung in the smoke-exposed animals when compared to filtered air-exposed control animals -- consistent with sidestream smoke **not** being carcinogenic under the test conditions -- even though they did report positive results for molecular biomarkers. Thus, these data suggest that the relationship of mutagenicity to "carcinogenicity" is not clear-cut.

In another study, Nikula, et al. (1995), investigated the inhalation carcinogenicity of two substances that were essentially equivalent, except that one contained mutagens and the other did not.[142] If mutagenic properties were relevant to carcinogenicity, it would be expected that the substance with mutagens would have

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produced a stronger carcinogenic response. However, as the authors noted, the two substances yielded "very similar" responses in the test system. Thus, these data, too, suggest that positive genotoxicity results cannot necessarily be correlated with carcinogenicity.

In its Proposed Rule, U.S. OSHA discussed a number of studies in which cigarette smoke or cigarette smoke condensate was tested in the Ames Salmonella typhimurium assay, and an increased mutation rate was reported. OSHA's inclusion of studies dealing with mainstream and sidestream smoke revealed the misconception pervading the Proposed Rule that ETS, mainstream, and sidestream smoke are equivalent.

Moreover, OSHA omitted at least one relevant study from this discussion in the Proposed Rule. In 1991, Bombick, et al., reported on a cellular smoke exposure technique using rat liver cells and the Ames Salmonella assay.[135] After a three-hour exposure using ETS at a concentration of 1.5 mg total particulate matter/m^3, the authors report:

> Using the neutral red cytotoxicity and Ames mutagenesis assays there were no differences observed in the ETS-exposed cells and their respective room air controls, indicating that ETS was **biologically inactive as tested**. (emphasis added)

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Studies have reported that various constituents and extracts of ETS collected from indoor air are capable of inducing mutations in the Ames assay.[143-149] However, the significance of such reported findings has not been established. Virtually all air samples, whether in the presence or absence of smoking, can be shown to be mutagenic in various bioassays. Indeed, many substances, including foods and other "natural" materials, have been shown to exhibit mutagenic and/or carcinogenic properties.[148]

Of relevance, Sonnenfeld and Wilson report that sidestream smoke exhibits reduced activity as it ages and becomes diluted, that is, as it becomes ETS.[149] These authors report on an experiment in which cultured mouse fibroblast-like cells were exposed to mainstream or sidestream smoke of various ages. In this report, cytotoxicity (cell mortality) is used as a measurement of DNA damage sufficient to cause cell death. The authors write:

> Aging of SS smoke resulted in a rapid decline in the mortality generated by the smoke. As calculated from the linear regression curve, an increase in age of SS smoke of 30 [seconds] after generation would have resulted in a **total** loss of cytotoxic effects. (emphasis added)

Another area of relevant research comprises those studies that have compared the mutagenicity of body fluids of nonsmokers exposed to ETS and nonsmokers not exposed to ETS. Several of these

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studies report no significant difference in mutagenic activity.[129,130,134,136,150,151]

For instance, in a study by a team of German researchers, ten nonsmokers were exposed to ETS, generated by human smokers, for eight hours under two exposure conditions.[138] The two experiments were characterized by CO levels of 10 ppm and 25 ppm, respectively; according to the authors, both exposure regimes represent higher exposures than "real-life" situations. Elsewhere, they describe Experiment 2 as "far from being realistic,"[134] and bearing "no relation to a real-life situation."[136] In addition, the authors controlled for the effect of mutagens from the diet by keeping their subjects on a diet low in polycyclic aromatic hydrocarbons. Urine samples from both smokers and nonsmokers were tested in the Ames Salmonella assay. The authors report:[130]

> All urine extracts of ETS exposed non-smokers were found to be negative in the mutagenicity test when applying the [criterion] of Ames (doubling of spontaneous mutation rate).

Thus, even at exposure levels higher than would be expected on average, no increase in mutagenicity could be measured. These data do not support claims that ETS exposure is associated with an increase in mutagenic activity; moreover, because the samples come from exposed humans, the influence of physiological processes following exposure is indirectly taken into account.

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Citing the high variability of measures of urinary mutagenicity and questions about the relevance of increased urinary mutagenicity to cancer risk, the authors write:[136]

> The data suggest that nonsmokers in real-life situations take up very low doses of ETS constituents, and detoxification of the genotoxic substances inhaled is effective.

And:[137]

> Whether ETS exposure can lead to an elevated urinary mutagenicity is a matter of controversy. In most investigations no significant increase has been observed. . . .
>
> . . . The results of our investigations, as well as those of other authors, suggest that urinary mutagenicity, which would be a potential marker for ETS particle exposure, remains unchanged after ETS exposure.

The few studies reporting statistically significant increases in urinary mutagenicity among individuals exposed to ETS do not employ realistic levels of exposure to ETS, nor do they control adequately for the presence of mutagens in the diet of the study subjects.[152-154] For instance, in the Bos, et al., study, the exposure condition consisted of the smoking of 157 cigarettes over six hours in a room with "poor ventilation."[152] The relevance of such an exposure to "real-life" conditions is certainly

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questionable. With respect to diet, Bartsch, et al., acknowledge, concerning their study, that[154]

> Urinary mutagenicity is influenced also by dietary habits; although we collected information on diet, the dimension of the study (particularly as far as passive smokers are concerned) does not allow adequate statistical treatment of this potential confounding factor.

Other related studies have examined levels of various DNA changes in nonsmokers exposed to ETS.[131,138,151,155-158] Based on the data presented in these studies, nonsmokers exposed to ETS do not appear to exhibit increased DNA adduct formation, nor do studies report increased levels of chromosomal changes in cells of nonsmokers exposed to ETS. Discussion of these studies follows.

Collman, et al., collected data from 16 nonsmokers, 15 "passive smokers" (currently living with one or more smokers), and 13 current smokers, all women.[156] Sister-chromatid exchange (SCE) frequencies in lymphocytes (a type of white blood cell) were compared with and without coincubation with a chemical that ennanced the frequency of SCEs. Based on both assays, the authors report that "the frequency of SCEs in persons passively exposed to smoke was not higher than in nonsmokers."

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In a report by Husgafvel-Pursiainen, peripheral blood lymphocytes were examined for SCE frequency, a sensitive test.[157] This test uses cells **from the exposed individual**, rather than another organism, and also considers repair mechanisms, thus being a better representation of actual events at the cellular level. Study groups consisted of 12 smoking waiters and waitresses, 20 nonsmoking waiters and waitresses who were occupationally exposed to ETS, and 14 nonexposed office workers. The author reports that "[t]he mean SCE level in exposed non-smokers did not differ from that observed in the non-exposed group." Although no ETS measurements from the restaurants were reported, the author characterizes them as "heavily polluted," and the exposure as "long-term." This study, which reports data from persons exposed in a "real-life" situation, does not support claims of the genotoxicity of ETS.

Chromosomal aberrations (CAs) and SCEs were examined in peripheral blood lymphocytes from nine smoking waiters, 16 nonsmoking waiters exposed to ETS at work, and seven reportedly nonexposed nonsmokers by Sorsa, et al.[155] The authors report that "[n]o significant differences were seen between the groups or subgroups in the 2 parameters." Thus, no "genotoxic" effects could be detected in persons exposed to ETS at "real-world" occupational exposures.

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Holz, et al., report that DNA adduct levels were compared in monocytes (a type of white blood cell) of smokers and "heavily exposed passive smokers," who had been exposed in a chamber.[131] DNA adducts above background were reported in smokers; they disappeared in less than 40 hours. The authors report **no** above-background adduct levels in study subjects exposed to ETS.

In a study by Gorgels, et al., 50 self-reported ETS-exposed men ("passive smokers"; average 72.8 hours exposure per week) were compared with 56 self-reported low ETS-exposed men (average 5.1 hours per week).[158] SCEs in cultured lymphocytes were examined; the authors report that "[n]o difference was observed between low exposed non-smokers and the passive smokers." They conclude:

> Our results are in accordance with previous smaller studies in less homogeneous populations of non-smokers. These studies also failed to demonstrate even a tendency for an association between passive smoking and SCE levels.

Five male smokers, five male nonsmokers, and five male nonexposed nonsmokers were compared in Holz and colleagues' 1993 paper.[130] The endpoint examined was DNA single-strand breaks (SSBs), "considered to be an important parameter of genotoxic stress," in lymphocytes. The authors write:

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> All probands revealed measurable and varying SSB levels. Since DNA is an unstable molecule and estimated endogenous damage exceeds 100000 affected base pairs per cell per day, we assume that SSB base levels reflect unrepaired lesions. Active smoking caused an increase in SSBs in peripheral blood lymphocytes. This effect could not be found in passive smokers.

ETS exposure in this study consisted of five smokers each smoking 24 cigarettes in eight hours in an exposure chamber. This study provides no support for claimed genotoxic effects of ETS, even at a high exposure level.

This review of data from studies in which genotoxicity was assessed in persons actually exposed to ETS thus provides little, if any, support for the contention that ETS is genotoxic at levels encountered in workplaces and other indoor environments. As Doolittle stated in his submission to U.S. OSHA:[159]

> The hypothesis that ETS causes lung cancer is not supported by any of the available genotoxicity data. There is no evidence that ETS at or near ambient levels of exposure produces genotoxicity. The available published evidence comes to the opposite conclusion, namely ETS is not genotoxic.

G. Conclusion

In conclusion, the U.S. EPA Risk Assessment on ETS is not the definitive document portrayed by Cal/EPA. A large number of

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unresolved questions and uncertainties remain. Moreover, more recent data on exposure, animal inhalation studies and genotoxicity do not conclusively support U.S. EPA's conclusions, contrary to the statements made by Cal/EPA.

A large number of the submissions and articles critical of U.S. EPA's risk assessment are now part of the U.S. OSHA rulemaking record. U.S. OSHA also relied heavily on the U.S. EPA Risk Assessment, and commenters have called upon OSHA to critically evaluate its conclusions.

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SECTION IV: CAL/EPA'S TREATMENT OF THE EPIDEMIOLOGIC DATA OVERLOOKS A NUMBER OF IMPORTANT ISSUES

The following discussion will address a number of problems with Cal/EPA's treatment of the epidemiologic literature on ETS exposures and lung cancer in the Excerpt. A selection of relevant references is submitted as Appendix II.

A. Cal/EPA Fails to Acknowledge the Magnitude of the Limitations of Weak Association Epidemiology

Members of the scientific community have criticized the epidemiologic studies on ETS exposure and lung cancer for failing to consider certain factors, namely bias and confounding, that could affect the validity of the studies' risk estimates. The impact of such factors is particularly important in studies that, like these studies, report risk estimates that are "weak."[1,2] A weak association is represented by a risk estimate of less than 2.0 or perhaps even less than 3.0.[3-6] As Wynder notes:[3]

> [E]pidemiology has problems when the associations are of a low order of magnitude. In such instances, findings in the literature are, in general, inconsistent. . . .
>
> When risks are small, and especially when effects occur many years after their causes, detecting them, estimating their magnitude, and assessing their importance for the community in light of other relevant factors pose problems of study design, data collection, analysis, and interpretation which can be exceedingly difficult. (p. 139)

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Specifically, Gori (1995) comments about the ETS epidemiology:[7]

> The weak data on ETS are compatible with either a slight increase or a decrease of risk, but are impotent to certify either conclusion on a scientific basis. In the unlikely hypothesis that ETS were a risk, it would have to be small beyond detection because of the **inevitable and excessive noise to signal ratios of its epidemiology.** (p. 20) (emphasis added)

1. Cal/EPA's Discussion of the Potential Impact of Confounders in the Spousal Smoking Studies Is Incomplete

In the Excerpt, Cal/EPA essentially dismisses the possibility that confounding factors could contribute to the increased lung cancer risk reported in some of the spousal smoking studies. (p. 22) Cal/EPA cites only a few studies other than some of the spousal smoking studies themselves, in a review that is clearly incomplete and selective.

The use of spousal smoking status as a proxy for ETS exposure introduces substantial uncertainties into estimates of risk. Spousal smoking (marriage to a smoker) does not measure ETS exposure alone, but rather encompasses numerous variables that may be related to lung cancer risk.[8-12] These other variables, or

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confounding factors, are factors associated with the exposure being studied (here, spousal smoking status) **and** with an increased risk of the outcome under consideration (in these studies, lung cancer). Confounders may seriously affect estimations of risk purportedly due to ETS exposure.

As noted in a recent review:[13]

> Because the relative risks or odds ratios for human diseases reported to be associated with ETS exposure are typically no larger than the risks for confounding lifestyle factors, epidemiological studies of the association between ETS exposure and chronic disease should be designed to maximize data quality and statistical power.
>
> The ETS and chronic disease epidemiology studies conducted to date have not adequately controlled for all of the known confounding variables.

Few of the spousal smoking studies upon which Cal/EPA relies have taken even some of the many potential confounding factors into account. Therefore, the possible impact of confounders on risk estimates attributed to ETS is of paramount importance. Since actual ETS exposures are not measured in these studies, where reported spousal smoking status is used as a surrogate for ETS exposure, the risk estimates claimed for ETS are instead risk estimates for "marriage to a smoker." A number of

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potential lung cancer risk factors are associated with household smoking status.[14,15]

One of the most important potential confounders of the claimed ETS-lung cancer relationship is dietary differences between "smoking" and "nonsmoking" households. [A "smoking" household is one in which at least one person smokes; a "nonsmoking" household contains no smokers.] In epidemiologic studies, when a nonsmoking case or control reports marriage to a smoker, this would correspond to living in a smoking household.

Diet appears to be a true confounder of the spousal smoking-lung cancer relationship.[16,17] Diet satisfies the first necessary condition for being a confounder by being associated with lung cancer risk, as has been shown in a number of studies.[18,19] Second, dietary differences are associated with household smoking status; that is, the diets of nonsmokers living with smokers differ from the diets of nonsmokers living with nonsmokers.

Data show that smokers' diets are generally different from nonsmokers' diets.[20-28] In particular, the data, already suggestive of differences in diet according to household smoking status, have been dramatically strengthened by the addition of several recent studies.[8,29-32] Taken as a whole, the data strongly support an association between household smoking status and diet.

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Since diet is associated with lung cancer risk, it could well be a confounder in the spousal smoking studies which have failed to adequately account for diet in their analyses. The magnitude of the risks reported for some dietary exposures suggests that if only a few cases in a study had such exposures, it could have a significant impact on the risk estimates attributed to ETS exposure.

The following are brief comments on some of the recent literature that illustrates the correlation between diet and other lifestyle factors and household smoking status.

- A 1992 British study examined the consumption of fried foods, fats, fruits, vegetables, and sweets in smokers, nonsmokers, and exsmokers.[22] The authors reported that nonsmokers who live in smoking households "have a diet more like smokers," and that "diet could be an important confound in epidemiological studies of ETS." The authors also noted:

> Our analysis showed that non-smokers in smoking households ate fried food more often, more chips [french fries], less fruit in winter, more butter and less margarine high in polyunsaturates than non-smokers in non-smoking households. As we have pointed out, these habits are thought to increase the probability of cancer.
>
> These results suggest that it is wise to show caution when interpreting the disease patterns

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> of non-smokers in smoking households. Studies to date have failed to take into account the effect that differences in dietary and lifestyle behaviour between 'smoking' households and 'non-smoking' households may have on the incidence of cancer or heart disease.

In a 1993 paper, the same authors reported that, in addition to having higher intakes of saturated fats, never smokers living in smoking households consumed fats more often, drank more alcohol, and ate fewer root vegetables and cereal than did never smokers living in nonsmoking households.[23]

- Thornton, et al. (1994), examined 33 lifestyle factors in a survey of 9,003 British adults.[9] They report:

> [L]ike current smokers, passive smokers tended to be less educated; of lower social class; work in 'risky' occupations; drink more alcohol; do nothing to keep healthy; take longer before their first meal of the day; eat more fried foods and bread; eat less cereal, fruits, salads and low fat/polyunsaturated spread; drink more tea (but not more coffee); use more sugar in tea and coffee; not cut down on fatty foods; and be more neurotic and extrovert.

Thornton, et al., summarize their study as follows: "It has not perhaps been documented clearly before that smokers and non-smokers differ in so many lifestyle characteristics and that these are **nearly always in the direction of predicting a**

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higher risk of disease." (emphasis added) The authors also point out the importance of considering these factors, particularly when investigating weak associations in epidemiology, such as those claimed for ETS.

- Matanoski, et al. 1995[28], report on analyses of data from the NHANES I study, comparing nutritional and behavioral characteristics between nonsmoking women whose husbands smoked and nonsmoking women whose husbands were nonsmokers.[29] Nonsmoking women with smoking spouses were statistically significantly more likely to have an urban residence, to consume beef and the skin on poultry (both suggestive of increased fat intake), to drink alcohol, and to consume less of certain vitamins and other nutrients than did nonsmoking women married to nonsmokers. The authors summarize the importance of their findings as follows:

> [E]xposure to household tobacco smoke may not represent just a single exposure but a complex of factors, many of which, such as low vitamin intake and high alcohol intake, have been shown to influence the risk of cancer.

While the Matanoski, et al., study is referenced in the Cal/EPA Excerpt, the treatment is superficial and does not give adequate attention to the importance of this study's findings.

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- Emmons and colleagues (1995) also compared the diets of persons living with smokers and living with nonsmokers.[9] Their data further support the differences between smoking and nonsmoking households: persons living with smokers had less healthy diets overall, consuming more fat and less fiber, fewer fruits and vegetables, and fewer micronutrients than did nonsmokers who lived with nonsmokers. All the reported differences were statistically significant.

The importance of the potential impact of dietary confounding on reported estimates of lung cancer risk from the spousal smoking studies was described by Layard in a submission to U.S. OSHA's rulemaking record.[10]

> Dietary confounding alone could easily be large enough to explain the summary U.S. spousal smoking-lung cancer relative risk of 1.09 from [Layard's] meta-analysis of female studies.

Other data suggest the importance of numerous factors associated with cancer risk that may affect the reported results of the spousal smoking studies. Few, if any, of these risk factors have been considered in the epidemiologic studies on lung cancer, nor has U.S. EPA or Cal/EPA convincingly shown that these other confounders or independent risk factors for lung cancer may be discounted. These potential confounders or risk factors would not

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have to apply to all the spousal smoking studies; if they influenced only a few of the studies, U.S. EPA's and Cal/EPA's position would be seriously affected.

For instance, recent publications have reported the following lung cancer risk factors: body weight,[13] history of radiation therapy,[14] occupation,[15] previous lung disease[16] and family history of lung cancer.[17] Moreover, in some cases, the risks reported for these other factors are far in excess of the risk estimates suggested for spousal smoking.[14]

As pointed out in the Report of the Independent Working Group to the Health Care Committee of the Australian National Health & Medical Research Council (NH&MRC), while it is difficult for epidemiologic methods to detect very low relative risks, it is conceivable that factors having low relative risks could still impact the outcome of a study.[18] The Report notes:

> [T]hree factors, each having a relative risk of 1.1, if present together would induce (assuming no interaction positively or negatively between them) an observed relative risk of 1.33. It is impossible that confounding risk factors of this magnitude would be detected in the studies we have examined. Hence although these risk factors would not be detected, they could, in aggregate, produce a relative risk at least as large as that observed for exposure to ETS.

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Similarly, a 1992 review also addressed the possibility that confounding factors may have a combined effect on estimations of lung cancer risk:[26]

> In the absence of calculations of lung cancer risk when multiple factors apply, one can only speculate on the combined effect on an individual who, for example, might have a family history of lung cancer (RR = 2-4), lived in an urban area (RR = 1.2-2.8), worked in an occupation associated with elevated lung cancer risk (RR = 2 or more), was among the physically less active groups of the population (RR = 2) and, if a female, had the risk associated with a short menstrual cycle (RR = 2.2).

Until epidemiologic studies fully account for the possible impact of confounders and independent risk factors on estimates of lung cancer risk for spousal smoking, those risk estimates must be viewed with caution and carefully interpreted. **There is no indication in the Excerpt that Cal/EPA has done this.** As Gio Batta Gori, Sc.D., writes in a comment in the U.S. OSHA public record:[11]

> [A]ttributions of epidemiologic risk to ETS cannot be rationally sustained unless confounders and biases have been convincingly controlled, and adjustments have been objectively justified.

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2. Cal/EPA Has Inappropriately Dismissed the Potential Impact of Several Sources of Bias in the Spousal Smoking Studies

When interpreting the results of epidemiologic studies, the potential effects of biases must be considered. Bias refers to factors in the design, conduct, analysis, or interpretation of an epidemiologic study that erroneously lead to the appearance of a stronger or weaker association than in fact exists. Bias (and confounding) becomes particularly important when dealing with weak associations, i.e., risk estimates of 3.0 or less.[1,3-6,9]

One important type of bias particular to the spousal smoking studies is smoking status misclassification bias.[10,39] Smoking status misclassification occurs when smokers erroneously report themselves as nonsmokers in response to study questionnaires. None of the spousal smoking studies to date has been able to discount smoking status misclassification as a potential source of bias.[10]

It has been implied that the Fontham, et al., study[11] is superior to the other spousal smoking studies because it had accounted for smoking status misclassification. This is an incorrect portrayal. While Fontham and colleagues did the best they could to exclude current active smokers from among cases and controls, based on cotinine measurements, cotinine does not allow

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the determination of past smoking status.[42] Moreover, Fontham, et al., measured cotinine in hospitalized cases. The vast majority of hospitals severely restrict smoking (in fact, accreditation requires that smoking be banned); moreover, many lung cancer patients who happen to be smokers stop smoking after diagnosis. Thus, the cotinine measurements in this study did not even give a good indication of present smoking status, let alone previous long-term smoking status. The smoking status misclassification rates portrayed by Fontham, et al., as accurate are, in reality, **not** representative of the true situation.[42]

Smoking status misclassification could have a dramatic impact on the reported risk estimates in the Fontham, et al., study, and in other spousal smoking studies. For instance, a recent Congressional Research Service (CRS) Report on ETS calculated that, in the Fontham, et al.,[41] and Brownson, et al.,[43] studies, smoking status misclassification rates of **less than 10 percent** alone would account for **all** the reported elevation in risk at the highest exposure levels, and that misclassification rates of **less than three percent** would mean that those risk estimates would not achieve statistical significance at the 95 percent level.[44] Such misclassification rates are certainly possible, according to a recent review of the literature on this subject.[45]

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In addition to smoking status misclassification, another potentially important source of bias in the epidemiologic studies on ETS is misclassification of disease status, i.e., diagnosis of lung cancer. In many of the spousal smoking studies, disease diagnosis is haphazard and incomplete. Even in some of the better-designed studies, the possibility that tumors appearing in the lung may have metastasized from other sites remains likely. None of the studies confirmed lung tumor diagnosis via autopsy.

In a recent study, Kaye, et al., reported that in "emotionally charged situations," misclassification of disease diagnosis could inflate cancer risk estimates by some 30 percent.[45] Self-reports of cancer were compared with medical diagnoses of cancer for two groups of people. One group lived in a community with a hazardous waste treatment facility (test population); the other (control population) did not. The risk estimate for malignant tumors for the test population **decreased by 31 percent** when the more precise medical diagnoses were used instead of self-reports of having had cancer. The authors conclude:

> This study demonstrates the importance of verifying reported cases of disease, even a disease as well defined as cancer, in emotionally charged situations such as living in communities surrounding hazardous waste sites. If reported cases of cancer had not been verified, it would have incorrectly

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> appeared that community A had almost twice the rate of cancer as community B and that an association existed between living in community A and having cancer.

Given the increasing public attention paid to ETS, and the claims that ETS exposure is causally associated with lung cancer, ETS could be considered an "emotionally charged" issue. While this might not affect the earlier studies on ETS, it is certainly of potential importance for studies conducted in the last few years.

B. A Number of Unresolved Questions Exist Concerning the Fontham, et al., Study, Rendering Its Interpretation Less Clear than Cal/EPA Portrays

Cal/EPA offers essentially no critical comment on the Fontham, et al., (1994) spousal smoking study.[11] In fact, Cal/EPA appears to favor this study, commenting that its reported results are "closest to" those reported by the U.S. EPA in its Risk Assessment on ETS. (p. 7, 19) Moreover, Cal/EPA claims that this study "successfully addressed" the many weaknesses inherent in the spousal smoking study design. (p. 27)

Despite the contentions to the contrary in the Cal/EPA Excerpt, the Fontham, et al., (1994) study is still subject to the same limitations as the other spousal smoking studies. A number of criticisms of the Fontham study have been submitted to the public record at U.S. OSHA.[12,46,47] For instance, Sears and Steichen listed

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nine major categories of problems with the Fontham, et al., study, which they characterized as "significant design and execution flaws."[2] These included:

> [T]he study population has no male representation and is not even representative of the nonsmoking U.S. female population, under-representing rural subjects and massively over-representing minorities (especially Asians);
>
> [T]he percentage of adenocarcinoma is unusually high, possibly a reflection of abnormal demographics in the study population;
>
> [T]he phenomenon that urinary cotinine analyses fail to detect active smoking cases only, suggests that misclassification is more prevalent among the lung cancer cases than among the controls, leading to an inflated relative risk point estimate;
>
> [T]he failure to promulgate the use of colon-cancer controls to account for recall bias results in over-estimation of risk;
>
> [T]he use of frequency-only matching within age categories, combined with the high sensitivity of cancer incidence to age differences, likely introduces a bias resulting in inflated estimates of risk;
>
> [T]he categorization of individuals by broad race groupings fails too account for important lifestyle differences, especially among the large Asian subset of this study;
>
> [T]he inability of the standard linear logistic regression approach to fully account for strongly-coupled confounding variables . . . results in inaccurate estimation of risk;
>
> [T]he non-independence of the spousal-, workplace- and social-exposure study subpopulations forces Fontham's workplace

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> relative risk estimate to include potential contributive effects (including confounding) from both spousal and social exposure.
>
> [T]he absence of dose from the 'risk equation' necessitates reliance upon the recall of exposures that may have taken place decades earlier, often by a surrogate respondent.

Moreover, recent publications have raised additional questions about the Fontham, et al., study.[35,48,49] Perhaps the most serious questions about the study are raised in a post-hearing brief submitted to the U.S. OSHA record that points out what appears to be a significant misinterpretation.[49] In that submission, William Butler focuses on the data in the Fontham, et al., study concerning women who reported both childhood and adult ETS exposure. Fontham, et al., originally presented risk estimates for adult ETS exposure regardless of childhood ETS exposure status.

Butler divided the study subjects into four categories: (i) neither childhood nor adult ETS exposure (the reference group); (ii) childhood but not adult exposure; (iii) adult but not childhood exposure; and (iv) both childhood and adult exposure. He calculated a statistically significantly **negative** lung cancer risk for women with childhood but not adult exposure (OR = 0.35, 95% CI 0.12-0.99), which, he proposes, is a result of some bias in study design or data collection. Butler states that "Fontham et al.'s

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failure to mention this fact makes their analysis incomplete and their interpretation misleading."

Fontham, et al., included cases and controls from the group with the decreased risk in the comparison group for their analysis of adult lung cancer risk. According to Butler, the underlying bias became a "source of artificially inflated statistical estimates that incorrectly indicate a positive association between adult ETS exposure and lung cancer."

According to Butler, Fontham, et al., appeared to recognize that there was an "interaction" between childhood and adult exposure in the 1994 paper, but did not mention the bias identified in his reanalysis. He states that adjustment for the bias would be "expected to reduce" the risk estimates for spousal, household, workplace, and social ETS exposures. Butler then notes that Fontham, et al., have not provided sufficient data in their publications for these adjustments to be made, and suggests that the raw data from the study would be needed and should be released.

A number of other issues have been raised concerning this study. For instance, as noted earlier, despite the Fontham, et al., study's use of cotinine to assess current tobacco use, the authors themselves acknowledge that misclassification of ever smokers as lifetime never smokers is "problematic" because there is

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"no biomarker of lifetime tobacco use."[41] Moreover, only slightly more than half (54%) of cases had cotinine determinations, so that even recent active smoking was not excluded for nearly half of the cases.[41]

While the Cal/EPA Excerpt repeatedly stresses that the Fontham, et al., study is a **multicenter** case-control study, if the characteristics of the study population are examined, it is seen that the vast majority -- 81 percent and 86 percent, respectively -- of cases and controls come from two areas in California (Los Angeles and the San Francisco Bay area). Given this breakdown, the "multicenter" label is fairly misleading. Moreover, the authors provide no breakdown of the data by study center, and it is not possible to ascertain whether the reported risks were consistent across the centers. Heterogeneity in the data among study sites would argue against combining the data as was done by Fontham, et al.

Although Fontham, et al., state that "dietary cholesterol" was considered as a potential confounder, they do not provide sufficient information in the study publication describing this factor. Apparently, Fontham, et al., did not consider dietary saturated fat intake, recently reported by Alavanja, et al., to be associated with relative risks as high as 6.0 to 11.0.[42] The risk estimate was highest for nonsmoking women with **adenocarcinoma**; over

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75% of the cases in the Fontham, et al., study were adenocarcinomas. Alcohol consumption, another potential confounder, was also not mentioned. Recent studies have reported that smokers, and the persons living in their households, are likely to consume more fatty foods and more alcohol.[7,39]

The "adjustment" of the reported risk estimates is difficult to interpret, as the adjustment takes into account both study design variables (e.g., subject age) and potential confounders. For instance, the workplace risk estimate in the final report of the Fontham, et al., study was 1.12 before "adjustment" for several factors.[41] The "crude" risk estimate was not statistically significant. After adjustment, however, Fontham, et al., reported a statistically significant workplace risk estimate of 1.39. As was pointed out in submissions to the U.S. OSHA rulemaking record, the direction of the change was opposite what would be expected, and of a magnitude greater than many of the other adjustments in the paper.[12,46] Fontham, et al., do not provide adequate discussion of this unexpected outcome of adjustment, e.g., they do not explain which factor(s) had the most impact.

The above discussion illustrates that there are many unresolved issues concerning the Fontham, et al., study. For the Cal/EPA Excerpt to accurately reflect the nature of the ETS

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epidemiology, it should review the criticisms that have been raised concerning the Fontham, et al., study, and address them.

C. The Argument that a Number of the Spousal Smoking Studies Demonstrate a "Dose-Response" Relationship Is Scientifically Flawed and Cannot Be Used to Support Cal/EPA's Conclusions

In the Excerpt, Cal/EPA refers to reportedly increased risks in the "high exposure" subgroups from some of the spousal smoking studies and to claimed positive trends in risk estimates. (e.g., p. 19, p. 26) By so doing, Cal/EPA invokes the argument that these studies exhibit a "dose-response."

The "dose-response" argument is based on the claim that positive results of statistical tests for trend on epidemiologic data show that lung cancer risk increases with increasing reported exposure; that is, that those tests demonstrate that a "dose-response" has occurred. Those who adopt this argument further suggest that positive tests for trend satisfy the criterion for dose-response used in evaluating a causal relationship in epidemiology. As will be shown below, however, such claims about "dose-response" based on the epidemiologic studies do not withstand critical scientific scrutiny.

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1. **The ETS Epidemiologic Studies Contain No Actual Exposure Data; The Purported "Dose" Levels Cannot Be Assumed to Represent Dose at All**

The ETS epidemiologic studies do not measure actual exposures to ETS in the home or workplace. Rather, the studies rely on imprecise estimates based on individual recall. Other recent studies clearly illustrate that questionnaire responses are not accurate representations of actual ETS exposures, and are subject to bias.[50-52] As Wynder and Hoffmann[53] note:

> [I]n all ETS-lung cancer studies in 'never-smokers,' assessment of their lifetime exposure remains problematic as long as reliable biomarkers of uptake are lacking.
>
> Perhaps the greatest uncertainty about the epidemiological data is due to the unreliable information obtained by questioning volunteers in regard to their smoking habits.

H. Daniel Roth, Ph.D., noted in his submission to U.S. OSHA: "The ETS exposure data in the overwhelming majority of studies are far too weak for drawing epidemiological conclusions."[54] If the data are weak in terms of ETS exposure as a whole, they are likely even more limited when dealing with reported "specifics," such as levels of perceived exposure including the number of cigarettes smoked per day or the duration of a smoking history).

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An examination of the ETS-lung cancer epidemiologic studies reveals that they use a variety of definitions to "quantify" spousal smoking. For instance, some use number of cigarettes smoked per day without consideration of duration, while others use duration regardless of amount smoked. In addition, the studies do not use the same intervals to categorize amount smoked, e.g., the "highest exposure" in one study may be >20 cigarettes per day, while in another study, it might be >40 cigarettes per day. Thus, the highest exposure category in the first would be an intermediate category in the second. As noted in the 1995 CRS Report:[14]

> One implication of the potential disparity between the different types of exposure measurements is that combining risk [estimates from] several studies at the highest exposure levels probably yields misleading results. p. 31)

The authors of the CRS Report note elsewhere that U.S. EPA calculated just such an overall risk estimate for the highest exposure levels in the spousal smoking studies.

Given the limitations inherent in exposure estimates based on personal recall and recall by surrogate respondents, claims that the "exposure level" data in the ETS epidemiologic studies can be used to illustrate dose-response are based on a weak and inadequate foundation. Uncertainty exists with respect to both

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the actual exposures encountered by study participants and the degree to which study subjects may be misclassified according to exposure. These limitations alone suggest that it would be prudent to exercise great caution in making interpretations about "dose-response" from these data.

2. Claims that the Spousal Smoking Studies Exhibit Dose-Response Relationships Are Most Commonly Based on the Results of Statistical Tests for Trend; Such Tests Are Clearly Not Tests for Dose-Response and Should Not Be Interpreted as Such

Epidemiologists frequently imply that the results of a statistical test for trend (e.g., the Mantel extension test) provide evidence of a dose-response relationship. According to Maclure and Greenland, this overstates the evidence for dose-response, particularly if "dose-response" is considered to be a monotonic relationship, that is, one in which risk increases with each increment of exposure.[5] In fact, Maclure and Greenland point out:

> Tests for overall trend, such as the Mantel extension test, are **widely but erroneously** believed to be tests of the hypothesis that a monotonic dose-response relation exists -- that is, a relation in which risk continues to increase with each increment of exposure. (emphasis added)

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Statistical assessments of "trend" are **not** equivalent to developing a dose-response relationship.[18]

Maclure and Greenland point out several major statistical problems with applying the Mantel extension test to questions of dose-response.[55] First, they demonstrate mathematically that when there are few subjects in one of the exposure categories, the Mantel extension test is essentially algebraically identical to a test for overall association (the Mantel-Haenszel test). They explain the interpretation error that can arise from this misconception as follows:

> Numerous articles can be found in which authors conclude that the extension test for trend in risk over a trichotomous exposure, **when there are few subjects in one of the exposure categories**, is telling something extra. In fact, it is little more than a restatement of the results of the Mantel-Haenszel test of the collapsed dichotomous table. (emphasis added)

That is, mathematical considerations dictate that, if the subdivisions ("exposure categories") include one or more with few subjects, the Mantel extension test will have a positive result in a study reporting an increased risk when only two categories are considered. This is frequently interpreted as evidence of dose-response, when it is essentially only an artifact of the mathematics involved. The issue is relevant because a number of

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the spousal smoking studies have at least one category with less than 20 cases. Peter Lee has noted that dose-response relationships are frequently reported by those studies that report an association in the first place, an observation that would follow from the mathematical considerations described above.[32]

Moreover, Maclure and Greenland also note another limitation of the Mantel extension test: that it "**assumes** a particular dose-response model as part of its justification."[33] (authors' emphasis) They continue:

> [The Mantel extension test] is not a test of the appropriateness of that model: instead, it is a test for magnitude of trend given that the shape of the dose-response relation implied by the linear-logistic model is appropriate.

Similarly, J. Lee, et al., note that a number of possible dose-response models could be applied to the ETS-lung cancer data.[13] Noting the inconsistencies among the various studies in terms of the data reported, they point out that the analyst's choice of model to apply to the data can affect the conclusions:

> Any conclusions about dose response relationships should not simply be a reflection about the type of dose response model chosen by the analyst. If a linear model is fitted to data which truly have a threshold then it is highly likely that the

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> straight line will be judged to provide an adequate fit. **However this does not prove that the dose response is linear and any interpretations based on extrapolation could be quite erroneous.** (emphasis added)

Another problem with the Mantel extension test is that it does not take incremental increases in risk at **each** exposure category into account.[55] The nature of the test is such that a single increase in risk at **one** exposure category could be sufficient to produce a statistically significant test for trend. Specifically, Maclure and Greenland state:

> The heart of the dose-response hypothesis is found in the words . . . 'continuously increasing risk.' Not only does the first small dose of exposure influence risk, but additional doses further increase risk relative to the effect of the previous dose. All dose increments are hypothesized to have effects, not just one of them. **Because the extension test can yield a small p value if only one dose increment had an effect, it does not test the hypothesis of interest.**[56] (emphasis added)

Another limitation in using a test for trend was noted by J. Lee, et al., who pointed out that inclusion of the unexposed, or reference, group in the test for trend can result in a statistically significant outcome, even though the only difference is between nonexposed and exposed.[18] That is, the different exposure levels (the point of a dose-response analysis) may

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actually not be statistically significantly different from one another.

> [A]ssessment of this [dose-response] relationship should be independent of the assessment of a possible overall association, and hence . . . the **unexposed** group should be excluded from the analysis. The rationale for this exclusion is that RRs greater than unity for exposures greater than zero may not differ from each other and yet the test for trend will be significant. In this case a trend may not be present in the RR for exposures above zero. (authors' emphasis)

Thus, inclusion of the unexposed group in a test for trend can lead to an artifactual conclusion of a statistically significant trend. Moreover, because there is no error term associated with the control value (i.e., the unexposed group), the control value will have an infinite statistical impact on the reported relationship.

In a 1995 paper, Tweedie and Mengersen evaluate "dose-response," taking into account "the possible confounding effect that inclusion of the unexposed group may have."[56] In agreement with J. Lee, et al., and with Maclure and Greenland, they note that "an observed dose-response relationship may be in fact simply evidence of overall association but not of increasing (or decreasing) risk with increasing dose."

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Tweedie and Mengersen illustrate this using hypothetical data,[46] where all the risk estimates are equal at different dose levels, an exercise also carried out by Maclure and Greenland.[55] Tweedie and Mengersen also use actual data from one of the spousal smoking studies, where "we see a raised relative risk but no discernible increase of effect with increasing dose," to illustrate the same point. A "raised relative risk," as discussed later in this section, may well be due to the effects of bias and confounding; because of the nature of the test for trend, the spuriously elevated risk can lead to an improper claim of "dose-response."

Similarly, the authors of the CRS Report comment that, of the spousal smoking studies they reviewed,[44]

> All of the trend analyses include zero exposure. If the trend was linear down to zero exposure, then including that level in the trend analysis would yield the same results as when excluded. If there was a threshold effect, then a trend test which included the zero exposure level might show a trend even if an analysis which included only exposures above zero did not show such a trend. **In other words, a sharp rise at some exposure level above zero could incorrectly be interpreted as a dose response trend over all exposure levels.** (emphasis added) (p. 29)

So, if an elevated risk is reported (overall or for one exposure category), and if the nonexposed group is included in a

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test for trend, the result of the test will be statistically significant, even though risk does not increase with each increment of "dose." Thus, the use of a test for trend does not reflect dose-response, but simply the fact that, mathematically, the test is being "driven" by some reportedly elevated risk.

3. Claims of Dose-Response in the ETS Lung Cancer Studies Reflect an Increased Risk at the Highest Exposure Levels; Such Risk Estimates Do Not Reflect ETS Exposure, But Rather the Influence of Other Factors Associated with Spousal Smoking

The preceding discussion illustrates that a single elevated risk estimate can drive a so-called dose-response assessment, when a test for trend is inappropriately used to reach a conclusion about dose-response. This explanation could certainly account for the "positive" trends reported in the ETS epidemiology. As Paul Switzer, Ph.D., a statistician at Stanford University, notes:[67]

> [W]ithout the highest spousal exposure group there would be very few individual studies with statistically significant effects or significant dose-response relationships.

Why, then, are the risk estimates elevated at the highest reported spousal exposure levels? The most likely explanation relates to the fact that the elevated risk estimates are the result

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of unexplained bias and confounding. For instance, Springall writes in his submission to U.S. OSHA that data exist suggesting that spousal concordance (the tendency for smokers to marry smokers) increases with the amount smoked and therefore, "an enhanced tendency to a false dose-response exists in the presence of [smoking status] misclassification."[42] As smoking status misclassification has been adequately excluded in **none** of the spousal smoking studies, it is certainly possible that misclassification may contribute to the claimed dose-response relationships reported.[43] As noted earlier, only very small rates of smoking status misclassification could account for the risk estimates reported at high exposures by Fontham, et al.,[41] and Brownson, et al.,[43] demonstrating the clear potential for this form of bias to have an effect in this context.

Another source of bias, exposure misclassification, may also contribute to the reportedly higher risks at higher exposure levels. As the authors of the CRS Report note:[44]

> The more specific the question about exposure, the more precise the measure, **but the less accurate the recall.** That is, there is likely to be a very small error rate in reporting marriage to a smoker, but there could be a significant error in reporting actual amounts of exposure, such as numbers of cigarettes smoked by a spouse, particularly in the past. (emphasis added)

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Recall bias is also likely to produce an artificial dose-response trend in the spousal smoking data. Layard points out that cases may be motivated to try to explain their disease, which could lead to more complete recall or exaggeration of high exposure levels by cases than by controls.[38] This differential would produce apparently elevated risk estimates at high exposures. Layard also notes that, under this scenario, one would expect to see a reduction in risk (i.e., a risk estimate below 1.0) at low reported exposures, just as is seen when the data from the U.S. case-control studies on women are combined in a meta-analysis.

As noted earlier, the spousal smoking studies are not measuring ETS exposures, but rather are essentially addressing risks associated with "marriage to a smoker." Spousal smoking status carries with it a number of associated lung cancer risk factors that may be associated with amount smoked.[33]

As Layard writes:[10]

> [M]any potential confounders of reported spousal smoking-lung cancer associations, as well as smoking-status misclassification bias, are correlated with spousal smoking in a dose-dependent fashion, and such correlations could account for apparent dose-response trends.

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That is, if the effects of confounding factors and biases were indeed highest in the highest exposure group, the risk estimates for those groups would be expected to be larger. Thornton, et al., report data from their survey of risk factors in a British population that support such a relationship.[3] The highest prevalence of many of the risk factors (dietary and behavioral) they investigated was found in smokers of 20 or more cigarettes per day. This study also showed that persons exposed to ETS had higher prevalences of risk factors than did non-exposed persons. Thus, these data are strongly suggestive of a relationship such as that described by Layard.[1] As most studies of risk factor clustering have not focused on associations with amount smoked, this area requires further research. This explanation for the increased risks reported at higher spousal exposures nevertheless remains a viable one.

4. A More Rigorous Analysis of the Data Does Not Support the Dose-Response Claims that Have Been Made

In general, claims about dose-response are tenuously based on qualitative reviews of data from the spousal smoking studies. Moreover, they accept the equivalence between a positive test for trend and dose-response, which, as described herein, is inappropriate and misleading. The weakness of such claims is clearly demonstrated when Tweedie and Mengersen's more rigorous

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analysis of the "dose-response" data, published in the peer-reviewed literature, is considered.[86]

Tweedie and Mengersen's 1995 paper compares several techniques for assessing dose-response in epidemiologic studies. It includes both reanalyses of the dose-response data from the individual studies and a meta-analytic assessment. The authors comment that they "provide a more rigorous approach than the purely qualitative assessments which have often been used in the literature."[86]

As an initial step, Tweedie and Mengersen recalculate tests for dose-response for all the spousal smoking studies reporting sufficient data, using three alternate methods. The first (the Armitage method) simply tests whether the reported risks at different categories are significantly different. The authors note that its "major benefit" is that no assumption about the pattern of dose-response is made. (Since the shape of the dose-response, if any, is unknown, using models that impose a shape will influence the outcomes of the test.) The other two methods fit models, one exponential and one linear, to the data, i.e., each entails a different assumption about the nature of the dose-response. For each of the three methods, the authors perform one test **including** the unexposed group, and one test **excluding** the unexposed group.

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Tweedie and Mengersen report that when the unexposed group is **excluded**, for all three tests, only one study fits one of the models; **no other studies show a statistically significant "dose-response."** Conversely, depending on the model, when the unexposed group is included, anywhere from two to all of the studies are statistically significant (at least at the 10 percent level). Thus, these analyses illustrate the sensitivity of conclusions about dose-response to the method of analysis.

In addition to the individual-studies analysis, Tweedie and Mengersen analyze the data from the case-control studies using meta-analytic techniques. Again, three models are used, and the results differ by model and, particularly, by whether the unexposed group is included.

In conclusion, Tweedie and Mengersen note that "a simple assessment of point estimates -- without consideration of their accuracy as expressed by associated confidence intervals and without a more rigorous method of synthesising the results from individual studies -- can be quite misleading."

Overall, Tweedie and Mengersen conclude:[56]

> From the meta-analysis of studies of lung cancer and exposure to ETS in non-smoking females given here, our conclusion is that,

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despite a significant observed relative risk associated with overall exposure, there is little increase in relative risk with increasing dose. **One explanation that accounts for this somewhat unexpected situation, of an overall observed increased relative risk but little indication of positive dose response, is that some bias may be inflating all of the observed risks.** (emphasis added)

5. Conclusion: Claims About a "Dose-Response" Relationship Derived from the ETS Epidemiologic Studies Are Unfounded and Should Not Be Relied Upon by Cal/EPA

The foregoing discussion clearly illustrates that the claims that data in some of the epidemiologic studies on spousal smoking and lung cancer support a dose-response relationship are based on a misinterpretation of the use and meaning of statistical tests for trends. Positive trend results can be explained due to spuriously elevated risk estimates arising from uncontrolled confounding and biases. Moreover, the results of more appropriate, more rigorous statistical analyses reveal inconsistencies among and within studies in terms of "dose-response." Because so many questions remain, "dose-response" claims should not be used by Cal/EPA to support its analysis of ETS.

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Attachment C to Comments on Chapter 7 -- Carcinogenic Effects

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Attachment D to Comments on Chapter 7 -- Carcinogenic Effects

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